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A PERMEABILITY FACTOR RELEASED FROM GUINEA-PIG SERUM BY ANTIGEN-ANTIBODY PRECIPITATES

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MANY attempts have been made to identify the mediators of the increased vascular permeability of inflammation. Most of the work has been concerned with the liberation of histamine but, more recently, other substances such as 5-hydroxy-tryptamine, polypeptides and serum globulins have received attention (Spector, 1958). Nearly all the more recent work has dealt with inflammation induced by irritants, burns, releasers of active substances (such as dextran, egg-white, compound 48/80, etc), or with *in vitro* changes of normal serum, plasma or lymph. Little is known about the mediators of allergic inflammation : it is tacitly assumed by many authors that histamine alone is involved. However, hesitation in accepting the histamine hypothesis stems from the failure of anti-histamine drugs to inhibit certain types of allergic response (Brocklehurst, Humphrey and Perry, 1955). This failure is usually explained in terms of Dale's (1948) concept that intrinsically-liberated histamine is less accessible to antagonistic drugs. Rocha e Silva and Rothschild (1955) showed that cutaneous anaphylaxis in rats could still be obtained in animals from which histamine had been depleted by dextran, ovomucoid or compound 48/80. Again, Inderbitzin and Dobrić (1959) found that the pattern of changes in histamine content of skin at sites of passive cutaneous anaphylaxis resembled those at adjacent control areas of abdominal skin. They concluded that the skin-histamine released during cutaneous anaphylaxis could not have mediated the resulting inflammation. As an alternative hypothesis, the possibility that allergic inflammation is caused by other substances is equally tenable and should be explored.

Allergic reactions result from the interaction of antigen, antibody and body constituents and can take many forms. We have selected for study the acute inflammatory response induced by injecting antigen into the skin of sensitized animals. As a first step we have added antigen-antibody precipitates (Ag/Ab) to serum and studied the ability of the treated serum to cause a rapid and sustained increase in vascular permeability when injected intradermally. In order to simplify interpretation of results we have, as far as possible, restricted our

observations to a single species—the guinea-pig—and have found that, under certain specified conditions, the addition of Ag/Ab to guinea-pig serum leads to the formation of a permeability factor which is distinct from histamine or a histamine liberator. We refer to this factor as PF/P.

MATERIALS AND METHODS

Antigen-antibody precipitate.—Sandylop rabbits were immunized with alum-precipitated egg-albumin solution by repeated intravenous injections over a period of 8 weeks. The pooled antiserum was stored at −20°. Antibody N content was determined by the quantitative precipitation technique (Kabat and Mayer, 1948). The Ag/Ab was prepared at equivalence by adding a solution of crystallized egg-albumin (Armour) to a sample of the antiserum and incubating at room temperature for 1 hr. All precipitates were prepared freshly each day, and, before use, washed three times with saline and centrifuged.

Activation of serum.—Blood was obtained from the heart of normal guinea-pigs, of either sex, weighing between 300–800 g. Serum, obtained from this blood was always used on the day of collection. Each ml. of neat or diluted serum was added to a portion of washed Ag/Ab containing 0·05 mg. of protein N as determined by the micro-Kjeldahl method. The Ag/Ab was suspended in the serum by shaking and the mixtures allowed to stand on the bench for 1 hr. before being tested for their ability to increase capillary permeability.

Increased capillary permeability.—All the experiments were done in guinea-pigs. It was essential to use healthy animals given an adequate source of vitamin C. They were fed on crushed oats and cabbage and in addition were given 4 mg. of vitamin C by mouth twice weekly. Size and sex were less important—satisfactory results were obtained with both males and females in the weight-range 200–500 g.

The animals were shaved with electric clippers and a solution of pontamine sky blue (5 per cent in 0·45 per cent saline) 0·15 ml./100 g., given into an ear vein 2–5 min. before the intradermal injection of the solutions under test. The intradermal injections were made with a 20 gauge needle, bevel downwards and introduced into the skin as superficially as possible. No anaesthetic was used—the animals were held by an assistant. Pinching of the skin at the site of injection was avoided. Up to 10 injection sites were used on each animal and in many experiments the order of injection was randomized. The animals were killed by a blow on the head 15 min. after the intradermal injection and portions of skin containing the injection sites were removed and pinned, inside uppermost, on a cork mat. The maximum and minimum diameters of the blue areas were measured on the following day with the aid of dividers and a ruler calibrated in millimetres.

As a rule each group contained 4 control animals and 4 animals which received mepyramine (5 mg./kg. intraperitoneally 15 min. before the intradermal injection) to distinguish between the effects of histamine and those of other substances.

Histamine-release in vitro.—Three methods were used to test the activated serum for histamine-releasing properties. Guinea-pig ilea were mounted in oxygenated Tyrode's solution containing atropine 1 μg./ml.; guinea-pig lungs were perfused by the method of Rocha e Silva and Aronson (1952) and minced guinea-pig lungs were used according to Mongar and Schild (1953). Compound 48/80 gave the expected histamine-release in all these preparations.

Serum kinin.—Rat uteri and duodena were prepared by the methods described by Gaddum and Horton (1959). Serum kinin prepared from ox serum and trypsin contracted the uterus and relaxed the duodenum at 1 μg./ml.

Fractionation of serum.—Serum proteins were fractionated by chromatography on diethylaminoethyl cellulose (DEAE cellulose Eastman Kodak) having a specific adsorption of 44 (determined by the method of Peterson and Sober, 1956) and containing 0·88 m-mole ionizing groups/g. The cellulose was suspended in 0·01 M phosphate buffer, pH 8·0, equilibrated and the pH of the supernatant fluid adjusted to pH 8·0 by cautious addition of phosphoric acid. Columns were packed according to the method of Sober, Gutter, Wyckoff and Peterson (1956). Serum was equilibrated with the starting buffer (0·01 M phosphate buffer pH 8·0) by dialysis for 24 hr. at 2°. The small precipitate of euglobulins which resulted from this dialysis was separated by centrifugation and the supernatant fluid was then applied to the column. The entire separation was performed in the cold room at 2°. A cone-sphere apparatus

was used to give a gradient for elution (Fahey, McCoy and Goulian, 1958). The spherical mixing vessel contained 1 litre of starting buffer and the cone held 500 ml. 0·3 M NaH_2PO_4 pH 4·3. The eluate was collected in fractions of 10 ml.; optical densities were measured at 280 mμ. Fractions were bulked according to the elution pattern; the bulked fractions were dialysed, freeze-dried and examined by paper electrophoresis.

RESULTS

Formation of a permeability factor (PF/P) by the addition of Ag/Ab to serum

The intradermal injection of neat or diluted guinea-pig serum into guinea-pigs produced an increase in capillary permeability only slightly greater than that produced by saline. On the other hand, sera containing Ag/Ab (equivalent to 0·05 mg. protein N per ml.) produced an increase in permeability comparable to that produced by 0·5 μg. of histamine. Furthermore, the effect of the treated serum was only slightly inhibited by 5 mg./kg. of mepyramine given intraperitoneally 15 min. before the intradermal injection : this dose completely inhibited the effect of histamine. Table I shows the results of such an experiment in which groups of 4 guinea-pigs were used.

TABLE I.—*Activation of Guinea-pig Serum by Antigen-antibody Precipitate : Effect of Mepyramine*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
Neat serum + Ag/Ab . .	16·6	19·3
1 : 10 serum + Ag/Ab . .	11·3	17·5
Neat serum . . .	5·2	7·3
1 : 10 serum . . .	5·4	6·4
Saline + Ag/Ab . . .	5·6	7·1
Histamine in saline (0·5 μg.) .	3·8	18·8
Saline	4·4	4·5

Properties of PF/P which distinguish it from other permeability factors

The mild inhibitory effect of mepyramine on PF/P appeared to rule out histamine as the main cause of increased permeability. No contraction of a guinea-pig ileum, suspended in 20 ml. of Tyrode's solution, was caused by 1 ml. of activated serum, nor did it release histamine from minced or perfused guinea-pig lung. The duration of increased permeability was greater with PF/P than with histamine as is shown in Table II. For this experiment groups of 4 animals

TABLE II.—*Comparative Persistence of the Permeability Increasing Effects of PF/P and Histamine*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)		
	Dye 60 min. after i.d. injection	Dye 30 min. after i.d. injection	Dye 2 min. before i.d. injection
1 : 10 serum + Ag/Ab .	6·1	15·3	15·9
1 : 10 serum . . .	5·1	4·2	3·8
Histamine (0·5 μg.) . .	3·0	3·1	18·1

received dye at various intervals after the intradermal injection. PF/P was still fully active 30 min. after injection whilst the effect of histamine was characteristically short-lived.

PF/P is not a kinin because it did not contract the rat uterus or relax the rat duodenum. Furthermore, the activity was neither dialysable nor extractable with hot ethanol. Holdstock, Mathias and Schacter (1957) have shown that a kinin-like substance is formed when guinea-pig serum is diluted. This kinin is unstable, being rapidly destroyed by the peptidases of serum. A 1 : 10 dilution of guinea-pig serum in saline was prepared and incubated in a water bath at 37°. At various intervals 1 ml. amounts of the dilution were added to an isolated organ bath containing a rat uterus. The period elapsing between addition of the diluted serum and contraction of the uterus was noted. This is a measure of the amount of kinin present. The experiment was then repeated using 1 : 10 serum containing Ag/Ab (0·05 mg. protein N per ml.). Table III shows the mean result of 4 such

TABLE III.—*Lack of Effect of Ag/Ab on the Formation and Destruction of Kinin in Freshly-diluted Guinea-pig Serum*

Time after dilution (min.)	Lag period before contraction of uterus	
	Dilution + Ag/Ab	Control dilution
0	>2 min.	>2 min.
2	1 min.	1 min.
10	40 sec.	40 sec.
20	1 min. 40 sec.	1 min. 40 sec.
30	>2 min.	>2 min.

experiments. Ag/Ab did not alter either the rate of formation or the rate of destruction of kinin.

Contact with a glass surface appears necessary for the activation of some permeability factors (Armstrong, Keele, Jepson and Stewart, 1954). This is not so for PF/P since plasma obtained from blood collected in a siliconed syringe and transferred to a polythene centrifuge tube in the presence of 1 : 10,000 heparin was still activated by Ag/Ab in a similar manner to serum obtained from blood allowed to clot in glass (Table IV).

TABLE IV.—*Formation of PF/P in Guinea-pig Plasma Collected in a Siliconed Syringe and Transferred to a Polythene Tube in the Presence of* 1 : 10,000 *Heparin*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine 5 mg./kg. i.p.	Controls
1 : 10 serum + Ag/Ab	12·5	17·4
1 : 10 plasma + Ag/Ab	11·4	16·4
1 : 10 serum	6·3	7·6
1 : 10 plasma	5·1	6·9
Saline + Ag/Ab	4·9	7·1

Some other properties of PF/P

Only preliminary studies have been made of the conditions necessary for optimal production of PF/P. Continued presence of Ag/Ab is not required for activity since its removal by centrifugation just before injection did not affect the result (Table V). Serum heated at 60° for 30 min. was not activated by Ag/Ab (Table VI).

TABLE V.—*Effect of Removing Ag/Ab from Activated Serum*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
(1) 1 : 10 serum with Ag/Ab still present	14·3	18·6
(2) 1 : 10 serum : Ag/Ab removed by centrifugation	15·2	18·4
(3) 1 : 10 serum	9·6	10·4
(4) Saline with Ag/Ab still present	5·8	7·9
(5) Saline : Ag/Ab removed by centrifugation	4·3	5·8
(6) Control saline	3·6	5·6

All tubes were allowed to stand for 1 hr. at room temperature. Tubes 2 and 5 were then centrifuged for 5 min. of 4000 r.p.m., the supernatants removed and 0·1 ml. amounts injected into guinea-pigs.

TABLE VI.—*Lack of Formation of PF/P in Heated Serum*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
1 : 10 serum + Ag/Ab	15·8	17·1
1 : 10 serum heated 60° 30 min. + Ag/Ab	6·5	10·9
1 : 10 serum	6·0	8·6
1 : 10 serum heated 60° 30 min.	3·3	6·3
Saline + Ag/Ab	3·8	5·2

Activation was inhibited by isotonic buffers prepared from citrate, phosphate, barbiturate, acetate and tris (hydroxymethyl)aminomethane.

When serum was diluted 1 : 5 with saline and allowed to stand 1 hr., then Ag/Ab added and the mixture allowed to stand for a further 30 min., subsequent dilution to 1 : 10 with isotonic phosphate buffers (pH 6·8 and 7·4) did not inhibit PF/P. It seems therefore that phosphate inhibits production of PF/P but not its activity.

Activation was inhibited by heparin at 0·2 mg./ml. or more. Neither Soya bean trypsin inhibitor (1 mg./ml.) nor 10^{-3}M-di-isopropylphosphofluoridate (DFP) inhibited activation.

At the moment little is known about the specificity of Ag/Ab but an effect similar to that of PF/P was produced by the addition of zymosan (1·5 mg./ml.) to serum (mean lesion diam. 11·9 mm. in animals dosed with mepyramine and 14·9 mm. in control animals), but not by kaolin (25 mg./ml.) (mean lesion diam. 5·5 mm. in both groups).

Spector (1957) has shown that inflammatory pleural exudates, collected 24 hr. after intrapleural injection of turpentine in rats, fail to increase capillary permeability when injected intradermally, but can be induced to do so by lung mitochondria. A similar exudate obtained from guinea-pigs was inactive but became active after the addition of Ag/Ab (Table VII).

TABLE VII.—*Activation of 24 hr. Pleural Exudate by Ag/Ab*

Substance injected (0·1 ml. intradermally)	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
24 hr. exudate . . .	7·5	7·9
24 hr. exudate + Ag/Ab .	12·7	17·9
Ag/Ab in saline . . .	5·3	6·1

Activation of serum by acid

Experiments designed to test the effect of pH changes on the formation of PF/P by the use of conventional buffers were precluded by the inhibitory action mentioned above. Consequently an attempt was made to study this effect by addition of acid or alkali, relying on the buffering power of the serum itself. This attempt was also frustrated since acid alone without the intervention of Ag/Ab led to the activation of guinea-pig serum. A sample of guinea-pig serum was diluted 1 : 10 with saline, allowed to stand 1 hr., adjusted to various pH values with hydrochloric acid (0·01 N) or sodium hydroxide (0·01 N), allowed to stand a further 20 min., neutralized and then injected intradermally into blued guinea-pigs. Those sera which had been at a pH of 5·5 or less caused an increase in permeability similar in size and intensity to that produced by PF/P. Sera which had been at pH values between 6·0 and 11·0 were inactive (Table VIII).

TABLE VIII.—*Formation of Permeability Factors in Serum by Acid*

Initial pH of serum	Mean diameter of blue lesion (mm.)	
	Recipient animals dosed with mepyramine (5 mg./kg. i.p.)	Controls
5·0 .	Not done	16·0
5·5 .	9·8	11·8
6·0 .	5·0	6·2
7·0 .	4·0	4·0
8·0 .	4·0	4·0
9·0 .	4·0	4·0
11·0 .	4·0	4·0

Fractionation of serum on diethylaminoethyl cellulose

In an attempt to find the constituent(s) of guinea-pig serum necessary for activation by Ag/Ab, a sample of serum (37 ml.) was fractionated by chromatography on diethylaminoethyl cellulose. Eleven fractions were obtained from the

chromatogram (Table IX, Fig.). However, when the serum was reconstituted from these fractions together with the euglobulins it was found to increase capillary permeability in guinea-pigs without the addition of Ag/Ab. The three main fractions, γ-globulins, α_2-globulins and albumins were dissolved in saline and investigated separately by intradermal injection. The albumins were inactive, but the α_2-globulins gave a marked response at 0·6 mg./ml. and the γ-globulins at 0·1 mg./ml. (Table IX).

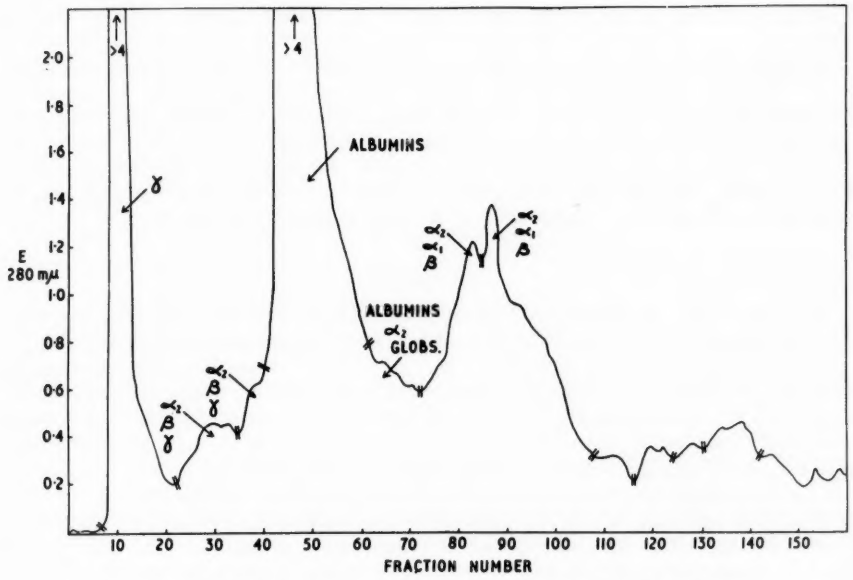

FIG.—Chromatography of guinea-pig serum on diethylaminoethyl cellulose.

TABLE IX.—*Chromatography of Guinea-pig Serum on Diethylaminoethyl Cellulose*

16 g. diethylaminoethyl cellulose giving a column 15 × 2 cm.

Fraction	Weight (mg.)	Constituents	Amount injected (μg.)	Mean diameter of blue lesion (mm.)
1	265	γ-Globulins	10	12·7
2	70	α_2-Globulins (+ trace β-globulins)	—	—
3	31	α_2 and β-Globulins	—	—
4	825	Albumins	223	6·9
5	134	Albumin + α_2-globulins	—	—
6	54	α_1-Globulins with some α_2-globulin and β-globulin	—	—
7	217	α_2-Globulins with some α_1- and β-globulin	60	11·0

Fractions 8, 9, 10 and 11 all appeared to be denatured. They weighed 12, 27, 2 and 29 mg. respectively.

Recovery from column 70 per cent.

Smooth muscle stimulants produced from PF/P and guinea-pig tissues

As stated earlier, PF/P did not release histamine from minced guinea-pig lung. At the end of the experiment the lung extracts were tested on the ileum in the presence of mepyramine (1 μg./ml.). The extract from lung with 1 : 10 guinea-pig serum and Ag/Ab caused a delayed slow contraction. When 1 ml. of the extract was added to a 20 ml. bath containing a rat uterus, again a slow contraction was obtained reminiscent of that produced by bradykinin. No contraction was obtained from lung incubated with saline, 1 : 10 serum, Ag/Ab in saline or from activated serum similarly incubated in the absence of lung tissue.

A similar activity was obtained from the interaction of PF/P and guinea-pig skin. The site of injection of 0·1 ml. of 1 : 10 guinea-pig serum, activated in the usual way with Ag/Ab, was excised from a guinea-pig 5 min. after injection and the portion of skin cut into small pieces with scissors and mixed with 2 ml. of saline. One ml. of this extract again produced a delayed slow contraction of the rat uterus. Saline extracts of skin injected with serum alone or with Ag/Ab in saline were inactive. Further work is being done on this aspect.

DISCUSSION

Inflammation, as Menkin (1956) has said, is a complex vascular reaction. This complexity has not been simplified by the present work since we appear to have added yet another permeability factor to those already known. Increased capillary permeability is a constant feature of inflammation, whatever the inciting cause. It is also technically easy to measure. For these reasons it has been extensively studied by many workers (Spector, 1958). Species differences in the response to different mediators is great (Sparrow and Wilhelm, 1957). We therefore decided to restrict our observations, at this stage, to one species.

Our own interest lay in the inflammation resulting from antigen-antibody interaction to which the guinea-pig is particularly sensitive. The system we chose for study, namely addition of Ag/Ab to serum, is one that has been extensively used elsewhere. It therefore behoves us to compare our results with those of others. Guinea-pig serum itself contains several known permeability factors. Battisto (1957) found that among a number of samples of sera from different guinea-pigs a small proportion increased capillary permeability. We have encountered this phenomenon ; the activity was slight and when it occurred the experiment was rejected. Several facts show that PF/P is not a kinin ; it is not dialysable, extractable by ethanol, does not contract rat uterus or relax rat duodenum. Moreover, Ag/Ab did not affect either production or destruction of serum kinin formed by dilution of guinea-pig serum. The relationship of PF/P to the globulin permeability factor PF/Dil (Miles and Wilhelm, 1955) is not yet clear but certainly its mode of formation is different in that PF/Dil is formed by merely diluting fresh guinea-pig serum 1 : 100 with saline whereas PF/P can be formed in neat serum. Formation of PF/P in fresh serum also suggests a difference from PF/Age which only occurs in aged serum. To the known ways of producing permeability factors in guinea-pig serum we must now add treatment with acid at pH 5·5. The high permeability effect of the γ-globulins isolated from the DEAE-cellulose chromatography was entirely unexpected. However, Ishizaka, Ishizaka and Campbell (1959) and Ishizaka and Campbell (1958) have reported

that when rabbit serum is separated by zone electrophoresis on starch blocks it is possible " to separate non-specific irritating substance which occurs in the fast moving components of the γ-globulin of some serums ". It seems possible that the γ-globulin isolated from guinea-pig serum by DEAE-cellulose chromatography could have contained such a component. Alternatively, it is conceivable that changes in the native γ-globulin structure could occur during absorption on to the DEAE cellulose as a result of either dissociation of the electrostatic linkages between the protein and the cellulose, alteration in the number of charges on the protein, or both. The present evidence does not allow us to decide which of these explanations is correct. The point is receiving further attention.

Permeability factors are also induced by antigen-antibody complexes themselves, under certain conditions, for example when the latter are solubilized by excess antigen (Ishizaka *et al.*, 1959, Ishizaka and Campbell, 1958 and Cochrane and Weigle, 1958). Our Ag/Ab was prepared at equivalence and was not itself active: indeed activated serum retained its effect when Ag/Ab was removed. Addition of Ag/Ab to serum is claimed to activate anaphylatoxin; the activity inhibited by mepyramine in our own experiments may be due to this substance but there is still a large effect not inhibited by mepyramine.

Further evidence that only a small amount of anaphylatoxin was formed under the conditions of our experiments was provided by the failure of serum with Ag/Ab to release histamine from guinea-pig lung or ileum.

Table X summarizes the differences between PF/P and other known permeability factors.

TABLE X.—*Permeability Factors in Guinea-pig Serum*

Factor	Biological property: Relaxation of rat duodenum	Contraction of g.-pig ileum	Contraction of rat uterus	Formed in neat serum	Formed in fresh serum	Inhibition by mepyramine
Kinin	+	+	+	+	+	−
Anaphylatoxin	−	+	−	+	+	+
PF/Dil	0	−	0	−	+	−
PF/Age	0	0	0	+	−	−
PF/P	−	−	−	+	+	−

+ = Positive response.
− = No response.
0 = Unknown.

The interaction of antigen-antibody complexes with complement is, of course, well established. Recently, Osler, Randall, Hill and Ovary (1959) have shown that component 3 of complement participates in the production of rat anaphylatoxin. The first component of human serum complement (C′1) is activated by Ag/Ab to an esterase (Lepow, Ratnoff and Pillemer, 1956), which can be eluted from the Ag/Ab. This esterase is inhibited by DFP (Becker, 1956). The fact that DFP (10^{-3} M) did not inhibit the formation of PF/P makes it unlikely that C′1 esterase plays a part in the reaction. The possibility that a relationship exists between PF/P and the other components of complement is under investigation.

PF/P is formed when Ag/Ab is added to guinea-pig serum; the mechanism of its formation has not, however, been elucidated. Lack of inhibition by soya bean trypsin inhibitor suggests that it is not produced as a result of proteolysis. It is possible that a natural inhibitor to PF/P, present in serum is removed or

inactivated by Ag/Ab. Alternatively it is conceivable that the immune precipitates cause some change in a serum protein such that on either intradermal injection or incubation with lung tissue, an active substance is formed. This implies that PF/P (which is non-dialysable) is the precursor of a factor which increases capillary permeability and stimulates smooth muscle. All these possibilities are being actively pursued as is the relationship between PF/P and the permeability activity produced by acid and by fractionation on DEAE cellulose.

SUMMARY

A washed precipitate prepared from egg-albumin and anti-egg-albumin rabbit serum when added to fresh, neat or moderately diluted, guinea-pig serum leads to the formation of a factor (PF/P) which increases capillary permeability in guinea-pigs. The properties and mode of formation of PF/P suggest that it is different from other known permeability factors. Permeability-increasing factors are also produced by treating serum with acid at pH 5·5 and by fractionation on diethylaminoethylcellulose.

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STUDIES ON TRANSFERENCE OF BONE

II. Vascularization of Autologous and Homologous Implants of Cortical Bone in Rats

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Although comments on the blood vessels can frequently be found in the literature on bone grafting, it would appear that few experiments were particularly designed for the study of the vascularization of bone grafts. Chase and Herndon (1955) in a review of the literature on the fate of autogenous and homogenous bone grafts, consider that the vascularization of homografts occurs as rapidly as that of autografts. In contrast to this, an inferior vascularization of homografts has been reported by Kiehn, Cebul, Berg, Gutentag and Glover (1952), Siffert (1955) and Stringa (1957), but these studies do not permit conclusions as to its cause and consequences. Felts (1957) and Hammack and Enneking (1959) have suggested a connection with the homograft reaction. From our preliminary studies (Zeiss Nisbet and Heslop, 1958) it could be expected that the inflammatory response to homogenous bone grafts might impair their vascularity.

In the present experiment an attempt was made to obtain detailed information about the mode and degree of vascularization of both types of grafts during the first 14 days and also at later dates. The experiment was designed so that the results could be correlated with our previous histological study on osteocyte survival, osteogenesis and the host reaction to autologous and homologous cortical bone implants (Heslop, Zeiss and Nisbet, 1960). In this way it was hoped to learn more about the possible inter-relationship between vascularization and the other phenomena involved in the host response to bone implants. It was considered desirable to estimate the number, length and origin of the vessels in the grafts and submit the results to statistical analysis. Histological sections were too thin for this purpose and therefore thick cleared sections, prepared from injected specimens, supplied the material for numerical evaluation.

MATERIAL AND METHODS

Animals.—Young adult rats were used throughout the experiment. Wistar rats were used for the autologous series. In the homologous series Wistar rats received grafts from hooded donors.

Preparation of implants.—Two types of implants were prepared : tubular pieces of tibia 1 and 1·5 cm. in length, and longitudinally split tibial grafts 2 cm. in length. Measurements were made in both cases from the proximal tibial epiphysis. From both types of implant the periosteum and medullary tissues were thoroughly scraped with dental excavators and reamers.

Site of implantation.—The implants were inserted between the dermis and the panniculus carnosus muscle, and some of the split grafts were implanted intramuscularly. Each recipient Wistar rat received either one tubular or two separate split implants.

Time of excision.—Autologous and homologous tubular implants were excised at daily intervals from 1 to 14 days after implantation, and later biopsies were taken at 35 and 250 days. Split implants were excised at 2, 3, 7 and 14 days.

Number of specimens.—A total of 264 specimens was examined. Each day of investigation was represented by 6 to 8 tubular implants and some days by 4 to 6 split implants as well.

Method of injection.—The animals were anaesthetized with ether, decapitated and pithed and the thoracic aorta cannulated. In the early stages of the experiment an 0·4 per cent suspension of Berlin blue in distilled water was injected, at a pressure of 300 mm. Hg for 5 min. This pressure tended to produce capillary ruptures, and in the later tubular specimens and all the split specimens the technique was modified, 2 per cent Berlin blue in distilled water being injected at a pressure of 100 mm. Hg for 10 min. This latter technique was found to give comparable vascular filling with only negligible capillary rupture.

Preparation of specimens for vascular study.—After fixation and decalcification transverse rings 200–700 μ thick were cut free-hand from the injected tibial implants and adjacent soft tissues. The split implants were embedded in gelatine and cut into transverse sections 200 μ thick with the freezing microtome. All specimens were cleared by the Spalteholz method (1914). The implants were floated in oil of Wintergreen in a glass chamber on a microscope slide and examined at $\times$ 120 magnification.

Method of counting vessels.—Vascular penetration, as indicated by the presence of dye, was defined in 4 classes, *viz.*, I, just entering bone ; II, well into bone but not beyond the outer lamellar zone ; III, into the Haversian zone ; IV, penetration of the full thickness of the cortex. The implants were of approximately the same transverse dimensions, and since the depth of the specimen was measured in each case, it was possible to estimate the number of vessels of each class (above) per 100 μ length of implant. This method of counting was used for the specimens examined during the first 14 days.

The rich vascularity of the later implants made this method of counting impracticable. Therefore the vascularity was estimated by impression only as poor and patchy, poor and even, satisfactory and patchy, and satisfactory and even. Corresponding portions of 3 normal injected tibias were available for comparison with the implants.

Assessing completeness of vascularization.—Cutting of transverse sections from bone always creates a certain number of Class I and II vessels, but the proportion of these is fairly constant in normal specimens, and the ratio of Class I and II vessels to Class III and IV vessels—" short " vessels to " long " vessels—may be used as an index of the incompleteness of a vasculature. In implants an abnormally high ratio will indicate the presence of a considerable excess of " short " vessels which is indicative of an incomplete vasculature.

Preparation of specimens for histological examination.—Material was fixed in 10 per cent neutral formalin, decalcified in 7·5 per cent nitric acid and embedded in paraffin. Sections were cut at 5 μ and stained routinely with haematoxylin and eosin. Histological examination was carried out on the tubular specimens only.

" Proximal " and " distal " bone.—Throughout this paper the term " proximal " bone refers to sections from the proximal third of the rat's tibia, and " distal " to the sections from the middle or distal third.

RESULTS

Autografts

Proximal bone

Vascularization of the tubular autografts was largely brought about by a steady ingrowth of vessels from the host as shown by the regression curves of the numbers of vessels in each of the four classes during the first 14 days (Fig. 1, 2, 3 and 4). The regression curves shown in these four figures are those polynomials which, upon regression analysis, gave a significantly good fit with the experimental data, the only exception being the curve of Class III vessels in proximal homografts, for which $P = 0{\cdot}05$. A direct dependency of the vascularization on time is indicated. Having reached its climax on the 8th and 9th days, ingrowth gradually diminished, and subsequent extension of the vasculature was achieved as a result of further growth of those vessels already in

bone. These facts are illustrated by the drop in the curve of Class I vessels and the steeper slope of the curve of Class III vessels from the 8th day onwards (Fig. 1 and 3).

At 14 days the vascularization of the tubular implants was still far from complete. In assessing completeness of vascularization at this stage, comparison with normal bone was restricted to those vessels growing in from the periosteal surface, because the medullary cavity of the implants had not as yet become completely filled by granulation tissue. Consequently parts of the medullary surface of the implant were inaccessible to new blood vessels. It was therefore impossible

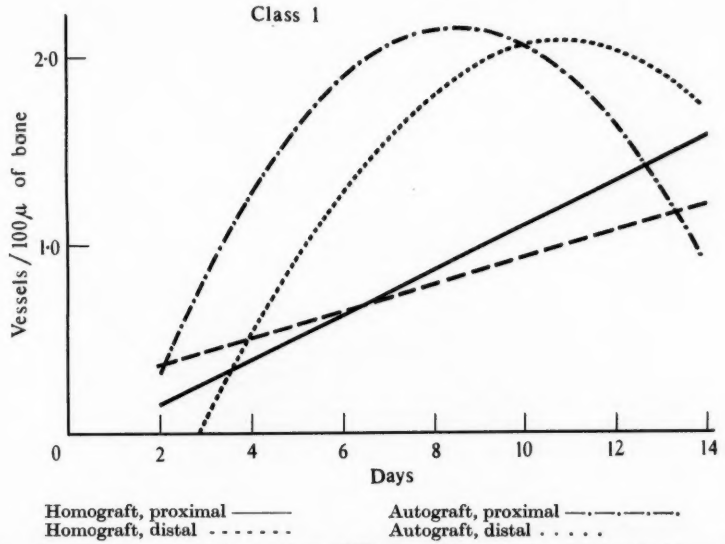

FIG. 1.—Regression curves of the numbers of Class I vessels (vessels just entering bone) over time. Note the difference in the slope of the curves for autografts and homografts during the first 5 days and the difference in the time of onset and climax in proximal and distal autografts.

to compare the numbers of blood vessels entering the medullary surface of tubular implants with those entering normal bone from this surface. The number of vessels of all classes entering from the periosteal surface were lower in the implants than in normal bone, the differences being significant in the majority. (For Classes I, III and IV, $P = 0{\cdot}001$, and for Class II, $P = 0{\cdot}02$–$0{\cdot}05$).

In spite of the differences in total vascularity between the implants and normal bone at 14 days, the ratio of " short " vessels to " long " vessels—(short vessels = Classes I and II ; long vessels = Classes III and IV)—was closely similar in both, the difference between the two means not being statistically significant. It would thus appear that new ingrowth of vessels at this stage was of a very low order, if present at all. These numerical data accorded well with the actual appearances in the injected cleared specimens. Some areas of the latter showed an approximately normal vasculature, while others were virtually avascular.

By comparison with the proximal tubular, the proximal split implants were much more evenly vascularized (Fig. 7 and 10), although their vasculature was still significantly sparser than that of normal bone. In contrast to the tubular specimens, the mean of the ratio of " short " vessels to " long " vessels in the split specimens was significantly higher than the normal mean. More detailed comparison between the vasculature of the split implants and normal bone showed that the " short " vessels group contained a preponderance of Class I vessels. The

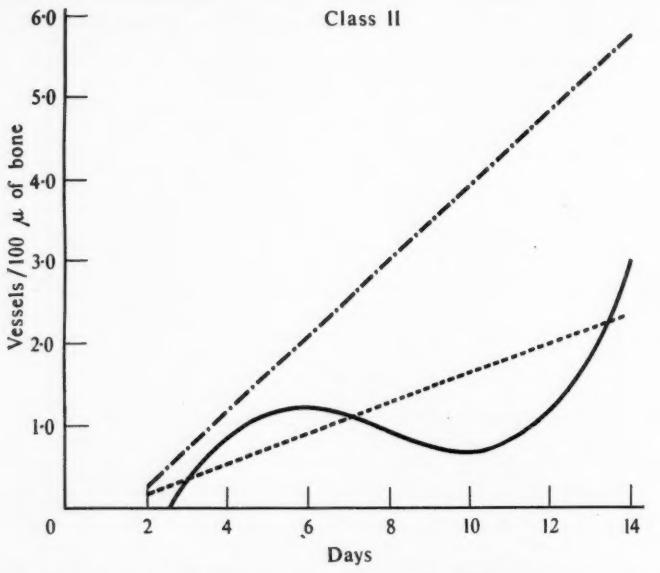

FIG. 2.—Regression curves of the numbers of Class II vessels (vessels well into bone but not beyond the outer lamellar zone) over time. The depression of the curve for proximal homografts is interpreted as resulting from the destruction of anastomosed graft vessels in the course of the homograft reaction. The difference in the slope of the proximal and the distal autograft curves is significant and demonstrates the slower vascularization of distal grafts. The number of Class II vessels in distal homografts showed no demonstrable relation to time.

mean number of Class II vessels was approximately normal. In both classes of " long " vessels the mean numbers were still significantly below normal. This evidence implies that at 14 days after implantation the vasculature of autogenous split implants was still incomplete, and that a considerable degree of ingrowth persisted.

In time the medullary cavity of the tubular grafts was filled with granulation tissue, which by 12 days was observed to have grown in for about 2·5 mm. Whenever present in this site its vessels showed numerous connections with those of the implant, and the appearance often suggested that the vessels in the granulation tissue partly originated from those of the implant. Vessels which had grown

through from the periosteal aspect branched into the medullary cavity (Fig. 8). The characteristic feature of the vasculature of the host reactive tissue at this stage was the great number of irregularly arranged capillaries. The progressive growth of this tissue seemed to facilitate the vascularization of those areas of the implants which had previously been avascular.

At 35 days the presence of new bone in the medullary cavity influenced the type and arrangement of the vessels in this region. There were irregularly

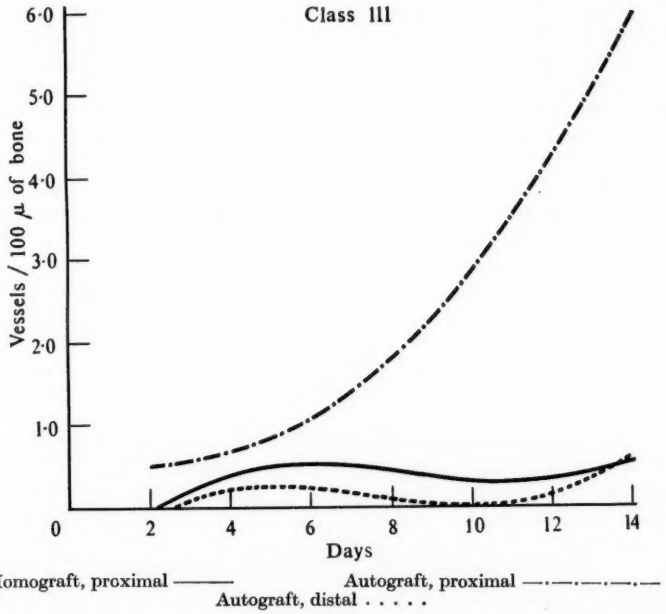

FIG. 3.—Regression curves of the numbers of Class III vessels (vessels reaching into the Haversian zone) over time. Note the steeper slope of the curve for proximal autografts after the 8th day, which is the time when the curve of Class I vessels reaches its climax. The course of the curve for proximal homografts is statistically not as well supported as that of the other curves ($P = 0{\cdot}05$). Its similarity in outline with the curves for Class II and IV vessels in proximal homografts increases the probability that it adequately reflects the actual situation. The number of Class III vessels in distal homografts showed no demonstrable relation to time.

distributed tufts of very fine capillaries which showed numerous connections with vessels in both the new bone and the implants (Fig. 11). At this stage 19 sections from 8 tubular implants were examined. The medullary cavities of all sections contained granulation tissue now modified as a result of osteogenesis. The vessels in this situation had apparently aided the completion of the grafts' vasculature. In 2 sections, however, the implant still contained small areas which were mainly avascular, although most of the remainder was satisfactorily vascularized. In 11 of the 19 sections, Class I and II vessels were absent, indicating a cessation of ingrowth. In the majority of these (9 out of 11) Class III and Class IV vessels

were present in satisfactory numbers. In 3 out of 19 sections the numbers of Class III vessels were poor or absent, but in one of these the numbers of Class I and II vessels were satisfactory, implying continued ingrowth. Class IV vessels were present in satisfactory numbers in all sections. Thus 17 out of 19 sections could be classified as satisfactorily vascularized.

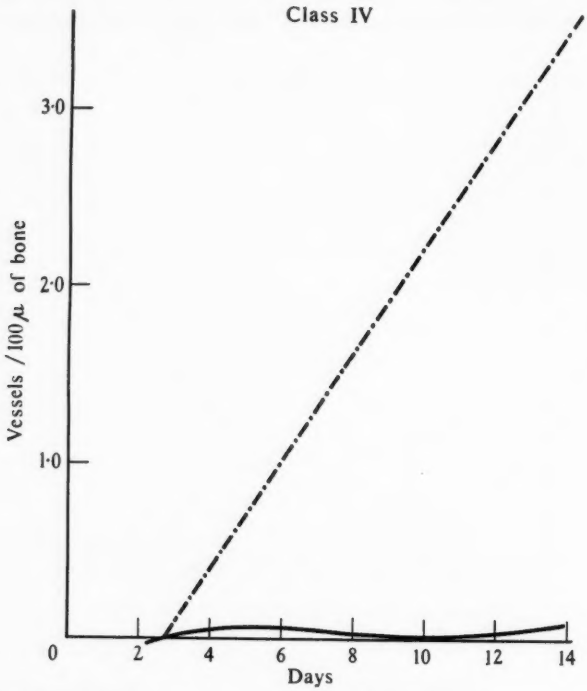

FIG. 4.—Regression curves of the numbers of Class IV vessels (vessels penetrating the full thickness of the cortex) over time. The superiority of proximal autografts over proximal homografts is obvious. The curve for proximal homografts again shows a decrease in number during the critical time of the homograft reaction. The number of Class IV vessels in distal grafts of either type showed no relation to time.

In spite of the fact that the vascularity of most autologous implants was classified as satisfactory at 35 days, it was still far from complete. Only 7 out of 19 sections showed an even and satisfactory vasculature throughout. There was a patchy distribution of Class I and II vessels in three sections, of Class III vessels in 9 sections and of Class IV vessels in 10 sections.

At 250 days 15 out of 16 sections from 7 tubular autografts had a satisfactory vasculature, and in 13 of these Class III vessels were evenly distributed. Only one section showed an irregular vasculature, which seemed to correspond with the irregular vascular pattern of the tissue inside the marrow cavity. Thirteen

out of 16 sections could thus be classified as both evenly and satisfactorily vascularized.

At this stage a comparison between the vascularity of the implants and normal bone was not feasible because of the considerable alterations in structure of the former due to extensive remodelling (Fig. 13 and 14).

Distal bone

The blood vessels in both tubular and split distal bone implants were found to be less consistently injectable than those of proximal implants. The same difference was noted between proximal and distal normal bone. This is indicated by the much wider scatter in distal than in proximal specimens (Fig. 5).

It was often noted that while the granulation tissue was in close contact with the concavities of the proximal implants, it was less closely attached to the distal implants. In addition, the quantity of granulation tissue around the distal specimens seemed to be less.

In the distal tubular autografts ingrowth commenced, and consequently reached its peak, 2 days later than in the proximal autografts (Fig. 1). The development and elaboration of the vasculature was likewise retarded, as indicated by the slow increase in Class II vessels (Fig. 2). The slopes of the regression curves of Class II vessels in distal and in proximal autografts differed significantly—$P = 0{\cdot}01$–$0{\cdot}001$ (Fig 2).

At 14 days the ratio of " short " vessels to " long " vessels was higher in the distal specimens than in the comparable proximal specimens, the difference from the normal ratio being significant in the split implants but not in the tubular implants.

In the distal tubular implants at 35 days, the numbers of Class IV vessels were classified as poor in 3 out of 16, and absent in 7 out of 16 sections. (Class IV vessels were present in satisfactory numbers in all of the corresponding proximal autografts). Class I and II vessels were poor or absent in 5 out of 16 sections as compared with 14 out of 19 in proximal bone. Class III vessels were poor in 3 cases. Only one of the distal sections had an even and satisfactory vasculature throughout. All the distal sections contained vascular tissue in the medullary cavity.

At 250 days distal tubular implants showed a poorer vascularity than proximal implants. Twelve sections from 6 specimens were examined. Class III vessels were present in satisfactory numbers in all. Their distribution however was patchy in 8 out of 12 sections. Class IV vessels were poor in number in 5 sections and patchily distributed in 6 sections. In one section the tissue in the medullary cavity was extremely poorly vascularized, although the implant contained a satisfactory number of Class III vessels of irregular distribution. Class IV vessels were poor in number. The vasculature in this case was obviously derived from vessels on the periphery of the implant.

Homografts

Proximal bone

In tubular implants ingrowth of vessels took place in a rhythm which was entirely different from that seen in autografts (Fig. 1–4). The increase in Class I vessels during the first 5 days was significantly slower, the difference in the slope

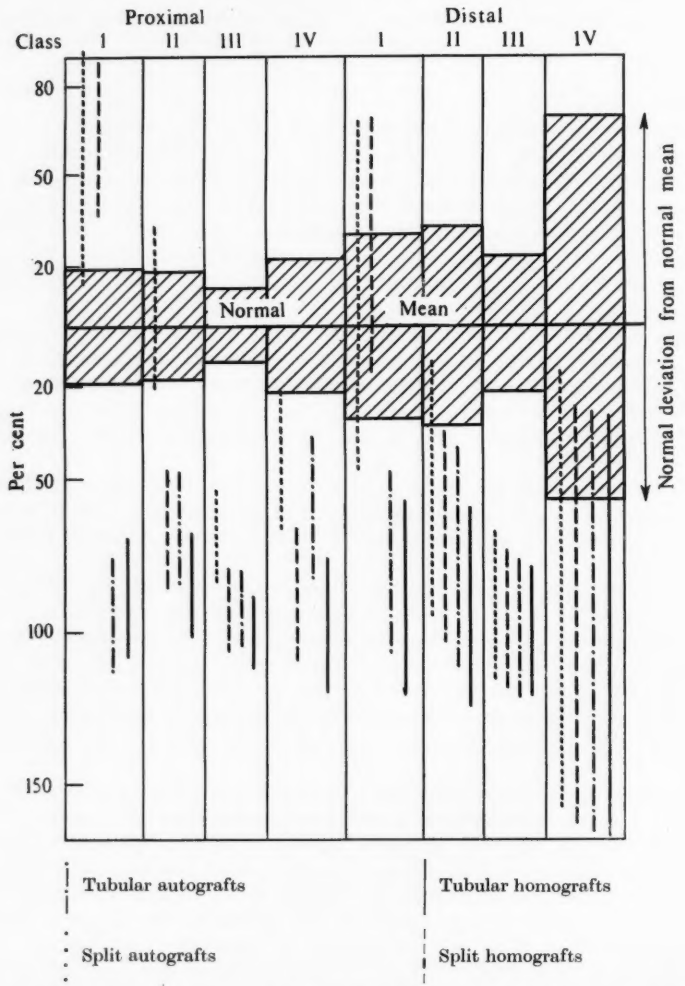

FIG. 5.—Vascularity of grafts 14 days after implantation, compared with vascularity of normal bone. The comparison is based on the 95 per cent confidence interval of the difference from the normal mean, expressed as a percentage of the normal mean. It shows the incompleteness of vascularity in all types of grafts at this stage, the superiority of split grafts over tubular grafts, of autografts over homografts and of proximal grafts over distal grafts. Note the superiority of the numbers of Class I vessels in split implants over the number of vessels in this class in normal bone, indicating a high rate of ingrowth in the implants. The less consistent injectability of distal bone as compared with proximal bone is reflected in the wider scatter of all observations relevant to the former.

of the regression curves being statistically significant ($P < 0{\cdot}001$). Class II vessels however were seen to increase at about the same rate as in autografts.

From the 6th to 12th day there was a relative decrease in the vascularization, or at least a diminished injectability. This is evident from the course of the regression curves of Class II, and III and IV vessels. At the same time, however, ingrowth was proceeding at the original rate, as the linear curve for Class I vessels indicates. The total decrease in vessels of Classes II, III and IV from the 6th to the 10th day was in the same range as the total number of vessels of these classes seen in homogenous implants 2 and 3 days after implantation. On the 10th and 12th day the numbers of vessels in all classes were significantly lower in the homogenous than in the autogenous implants.

At 14 days the homografts were in a phase of vascularization quite different from that seen in the equivalent autografts. The mean of the ratio " short " vessels to " long " vessels in both tubular and split homografts was extremely high and differed significantly from the normal. The individual counts in the various classes were significantly lower than normal, with the exception of the count of Class I vessels in the split implants, which was considerably in excess of normal (Fig. 5). The vascularity of both tubular and split homogenous implants was also significantly inferior to that of the corresponding autografts, again with the exception of Class I vessels, the counts of which did not show significant differences in the two groups.

The appearances of all proximal homografts at 14 days thus indicated that although ingrowth took place at a high rate, the vascularization was not only significantly inferior to that of normal bone, but also to that of the comparable autogenous implants (Fig. 7 and 9). This difference was more pronounced in the split implants than in the tubular specimens.

From the 5th and the 6th day onwards, well vascularized tissue was occasionally seen in the marrow cavity of the tubular implants and some of its vessels grew into bone. But often, even after 14 and 35 days, this tissue had no contact with the medullary surface of the bone. It resembled a disc with a radiating vascular pattern, occupying the centre of the marrow cavity. In some sections vessels were seen to pass from the medullary tissue into bone canals. In others, vessels penetrating the whole thickness of the implant communicated with vascular plexuses on both surfaces. The appearance noted in autografts (Fig. 8), suggesting that vessels in the medullary tissue originated from vessels in the implant, was never observed in homografts. Likewise, the tissue never seemed as closely attached to the medullary wall in homografts as it did in autografts. At 35 days, if the medullary tissue was present, it had a comparatively rich vasculature and the radiating pattern observed earlier was maintained (Fig. 12). At 250 days, however, its vasculature had been reduced to a few large vessels (Fig. 15).

By no means all the tubular homografts, however, contained tissue in their central cavities. At 35 days the medullary cavity of 4 of the 16 sections from 6 tubular implants contained little or no tissue. Three of these sections were particularly poorly vascularized. The marrow cavity of one section, which could be identified as the end section of the implant, was well filled with vascular tissue, and the bone was evenly and satisfactorily vascularized. This section was taken from the same specimen which supplied 2 other sections. One of these contained tissue in the marrow cavity with a vasculature similar to that of the

earliest stages and the other was completely devoid of such tissue. The bone in both was accordingly sparse in vascularity. These observations stress the importance of the vascular granulation tissue in the medullary cavity in the vascularization of the implants at this stage. It is interesting to note that even at this time the marrow cavity of the 1·5 cm. long homogenous implants was not yet completely occupied by host tissue.

In 14 out of the 16 sections from the 35 day implants, Class I and II vessels were poor or altogether absent. Class III vessels were classified as absent or poor in 8 of the 16 sections and Class IV vessels were similarly classified in 5 sections. The vascularity of the bone did not vary so much from specimen to specimen as from section to section, and seemed to depend on the amount and development of the vascular tissue in the marrow cavity. Seven out of the 16 sections had an even and satisfactory vasculature throughout. The vascular tissue in the marrow cavity was present in large quantities in all of these and appeared to make an important or even the major contribution to the vascularization of the bone.

At 250 days 3 of the 13 sections from 7 tubular implants did not contain any tissue in the marrow cavity. In 9 sections such tissue as was present contained only a few large vessels (Fig. 15). In one section the vessels in the tissue in the marrow cavity were radially arranged and grew into the bone. This implant contained a satisfactory number of evenly distributed Class I and II vessels along its inner circumference. Class III vessels were poor in number in 11 out of 13 sections and absent in the remaining 2, and were evenly distributed in only one section. None of the sections contained vessels of more than one class in satisfactory numbers and in even distribution at the same time. While 7 out of 16 sections examined 35 days after implantation were satisfactorily and evenly vascularized, none of the 13 sections examined at 250 days showed this degree of vascularization.

The vascular pattern of homografts at this stage was still comparable with that of normal bone, since no remodelling of the implants had occurred (Fig. 15).

Distal bone

The vasculature of distal homogenous bone, like that of distal autogenous and distal normal bone, was found to be much less readily injectable than that of proximal bone. This is illustrated by the wider scatter in the number of all types of vessels in all the distal implants as compared with the proximal implants, and the wider range of deviation of the normal specimens from the normal mean (Fig. 5).

In homografts the slower increase in Class I vessels in distal as compared with proximal bone was of a small order and statistically not significant (Fig 1). The vessel counts in Classes II, III and IV showed no demonstrable relation to time at all.

At 14 days the ratio " short " vessels to " long " vessels differed significantly from the normal ratio. It was higher in the distal specimens than in the comparable proximal specimens. It therefore seemed that the vascularization of homogenous distal bone resembled that of autogenous distal bone in being slower than that of proximal bone.

In the tubular homogenous implants the numbers of vessels in Classes II and III, but not in Class I, were significantly lower than those in the comparable

autogenous implants. Comparison of split homogenous and autogenous bone either with each other or with normal bone gave no clear-cut results 14 days after implantation.

At 35 days the marrow cavity of 7 out of 17 sections from 7 tubular implants did not contain vascular tissues, or at most some of recent development. In 3 out of these 7 sections the vessels were arranged in a radiating pattern and seemed to " point " to the openings of the bone canals, but did not grow in. The bone in these sections was particularly poorly vascularized, and in one it was completely avascular. In 9 of the 17 sections the bone was vascularized predominantly from the medullary aspect, *i.e.* through ingrowth of vessels from the vascular tissue in the marrow cavity. No specimens, however, were evenly and satisfactorily vascularized. In 12 sections Class I and II vessels were poor in number or absent. In 14 sections Class III vessels were similarly classified and in 15 sections Class IV vessels were likewise poor in number or absent.

At 250 days 2 of the 13 sections from 7 tubular implants did not contain any tissue in the medullary cavity. In 9 the tissue which was present contained only a few large vessels. In the remaining 2 sections the vessels of the tissue in the marrow cavity radiated from a large central vessel to bone canals and supplied the implants with a certain number of Class I and II vessels on the inner circumference. Class III vessels were poor in number in 7 out of the 13 sections and absent in the remainder. Here the vasculature consisted mainly of a small number of Class I and Class II vessels. Class IV vessels were absent in 2, poor in 9 and satisfactory in 2 sections. None of the sections could be classified as evenly and satisfactorily vascularized.

Possibility of anastomosis

In a quarter of the 2 and 3 day implants of all types some vessels were seen to penetrate the whole thickness of the tibial cortex (Fig. 16 and 17). They were found to measure up to 0·7 mm. in length. These appearances suggested that anastomosis could have taken place between host and graft vessels.

The role of granulation tissue in the vascularization of bone

The concavities on the surfaces of all the implants, and especially the medullary aspect of the split implants, were favourable sites for the formation of granulation tissue. It has already been mentioned that the presence of this tissue facilitated the vascularization of the implants. In the split implants at 7 and 14 days the vast majority of sections (44 out of 48 autografts ; 41 out of 48 homografts) showed a predominance of vessels in continuity with vessels on the medullary surface.

In 11 out of 18 sections of normal tibia more vessels were seen to be in continuity with the periosteal plexus than with the medullary plexus. The reverse phenomenon was seen in the remaining 7 sections. The differences between the two groups of implants and normal bone were statistically significant.

The abnormally high incidence of vessels connecting with the medullary plexus in the split implants did not seem to depend on the site of implantation. In the intramuscular implants this feature was seen in 20 out of 24 sections, and in the suprapannicular implants in 65 out of 72 sections (in the suprapannicular implants

the medullary surface was next to the muscle and the periosteal surface next to dermis). The difference between these two groups was not statistically significant.

Histological appearances of blood vessels

The blood vessels of both autologous and homologous implants showed essentially similar histological features in specimens excised during the 1st week following implantation. On the 2nd day the normal vascular structure was still easily identifiable in most of the Haversian and Volkmann canals. While many of these vessels contained no blood cells, a moderate number still showed recognizable red and white cells. Very occasional vessels on the 2nd day showed early degeneration. The initial degenerative change consisted of fusion of the blood cells into a structureless mass. The features of the vascular wall were not visibly altered at this stage. By the 3rd day, however, degenerative changes of some degree were present in the majority of the vessels, and loss of structure of both blood cells and endothelium was frequent. The majority of the Haversian and Volkmann canals thus came to contain structureless eosinophilic debris, sometimes with scattered nuclear remnants. A tiny number of apparently normal vessels remained towards the outer circumference of the implant. By the 4th day the proportion of degenerate vessels had increased still further although very infrequent normal vessels could still be found near the outer circumference of the implant. Of the numerous degenerate and normal blood vessels seen during the 2nd, 3rd and 4th days, Berlin blue was identifiable in only 3 vessels. One of these was in a 2 day homologous implant while the other 2 were in 4 day homologous implants. In the case of the latter 2 vessels, both showed the surrounding vascular wall to be degenerate. As there was spillage of Berlin blue into the tissues round one of the 4 day implants, the possibility of capillarity suggested itself. In the other 4 day injected vessel with a degenerate wall, however, there was no histological evidence to suggest that capillarity might have been responsible.

By the 7th day the majority of Haversian and Volkmann canals were quite empty in both series, although a small number of canals near the outer circumference of both types of implant contained endothelial lined channels. In a few cases it was possible to trace continuity of the latter with host vessels, although this had not been possible prior to 7 days in this type of preparation.

From the 10th day onwards the appearances of the vessels in the autologous implants differed considerably from those in the homologous implants. In the autologous specimens increasing numbers of the bone canals contained endothelium, and by the 10th day a few of these were seen to contain blood cells. The majority of vessels, however, contained endothelium only. On the 14th day the progressive increase in histologically normal vessels was maintained and small numbers of endothelial lined channels were visible near the inner circumference of the implant. The proportion of vessels containing blood cells was also increased. This progressive improvement in vascularization continued throughout the autologous series, until at 250 days the great majority of bone canals contained apparently normal vessels. The proportion of vessels containing blood cells likewise increased throughout the series.

In the homografts from 10 days onwards the pattern of vascularization was similar to that seen in autografts, although its rate was very much slower. By

the end of the 14th day the majority of Volkmann and Haversian canals were still empty and the few endothelial lined channels were restricted to the outer surface of the implant. By 35 days vessels were seen along the inner circumference of the implants, although their numbers did not approach the numbers seen in autologous implants at this stage. Even at 250 days vascularization lagged considerably behind the autologous specimens in so far as a larger proportion of bone canals still remained empty.

In addition to the slowness of vascularization, the small vessels near the homografts showed considerable histological abnormality in many cases. This consisted of hyaline thickening of their walls accompanied by some structural change in the surrounding collagen. These vascular changes were first visible in homologous implants at 35 days and were widespread at 250 days. These histological changes have been discussed elsewhere (Heslop *et al.*, 1960).

In all cases examined histologically the quantity of injected Berlin blue was small relative to the number of vessels in the implant which were apparently patent. Indeed in the 250 day injected specimens many of the vessels in bone contained blood cells among which no Berlin blue was visible. Despite the appearances in the implant vessels in these cases the vast majority of soft tissue vessels contained Berlin blue.

DISCUSSION

Validity of the injection technique as an index of vascularity

From the histological observations it might appear that the method of intra-arterial injection of a dye is not an adequate technique for vascular studies in bone, because only a small proportion of the actual number of vessels in the implants was seen to contain Berlin blue. Injected specimens of normal bone likewise showed relatively poorer vascular filling than did the adjacent soft tissues. Brookes (1958) found that filling of the vasculature of the rat's long bones with an injection mass was "adequate" when the filling of the vessels of the gut wall was complete. In spite of the lesser injectability of the vasculature of the long bones in the rat, it seems reasonable to assume that under standard conditions a similar proportion of the total vasculature will be injected in normal bone and also in bone implants. While the histological examination and the vascular counts on the cleared specimens were carried out independently, they gave essentially the same results, a fact which tends to support this assumption. It thus seems justifiable to consider the number of injected vessels as an index of the vascularity, and also as a reasonable means of comparison between different types of implant and normal bone. It is appreciated, however, that no exactly comparable controls are available for an experiment of this type.

Mode and time of vascularization

Under the circumstances of this experiment, where a tubular cortical bone graft was inserted in as narrow a pouch as possible between dermis and panniculus carnosus muscle, some degree of anastomosis occurred between host and graft vessels. This is illustrated by the number of Class IV vessels seen in the grafts 2 and 3 days after implantation. Also the decrease in vascularity in homografts at the time of the homograft reaction could be interpreted as indicating

that their vasculature partly consisted of donor tissue, which was destroyed in the course of the immune reaction.

There is strong evidence for the view that skin grafts are vascularized at least partly by anastomosis of grafts and host vessels (Taylor and Lehrfeld, 1953; Converse, Ballantyne, Rogers and Raisbeck, 1958; Converse and Rapaport, 1956; Nisbet, Zeiss and Heslop, 1958). From these findings in skin grafts, and from observations on grafts of embryonic bone to the chorio-allantoic membrane of chick embryos (Hancox, 1947), it appears reasonable to expect anastomosis of graft and host vessels to play at least some part in the vascularization of fresh bone grafts. The extent of anastomosis in bone grafts probably depends in the first place on how close a contact can be achieved with the host tissues. This will vary with the shape of the graft and the preparation of the graft bed. In addition, the original vascular pattern of the graft will be of importance. For example, in the case of pinch skin grafts in rats it was noted that extensive filling of the graft could be achieved as a result of anastomotic junction with one major vessel (unpublished data). On the other hand, with the bone implants used in this experiment, equivalent vascular filling could have been achieved only as a result of multiple anastomoses over a large surface area.

Further support for the possibility of anastomosis has been supplied by Ballantyne and Converse (1958), who found it to occur between vessels of a composite auricular graft and those of the chorio-allantoic membrane of the chick embryo, on which the grafts were implanted. They stated, however, that ingrowth of host vessels appeared to play an important part in the establishment of the definitive vasculature in this type of graft. The results of our present experiment lead to a similar conclusion in respect of bone implants. The time during which anastomosis could occur seemed to be restricted to the first 4 to 5 days, since it has been shown in this paper, and also by Ballantyne and Converse (1958), that degeneration of the original graft vasculature was widespread by the 4th and 5th day.

While allowing that anastomosis between graft and host vessels did occur, the evidence from this experiment, however, strongly suggests that these grafts were mainly vascularized by ingrowth of vessels from the host. Several facts combine to suggest vascularization by ingrowth rather than anastomosis: (1) the sparse vascularity of all implants at 5 days, (2) the steady increase of Class I vessels during the first 9 days in autografts and during the first fortnight in homografts, and (3) the obvious dependency on the completion of the vasculature of the autografts on time.

As regards the extent of ingrowth from the host and also the time of its onset, the vascularity of the adjacent host tissue appeared to be of secondary importance, whereas the presence of granulation tissue appeared to be essential. Areas of bone near a convex surface where granulation tissue was sparsely developed were vascularized poorly or not at all after 14 and 35 days—even in autografts. On the other hand, implants of split tibia, providing favourable conditions for the development of granulation tissue along the whole length of the medullary aspect, were evenly vascularized after a fortnight. It thus seems that since the conditions for anastomosis are not optimal, bone grafts of this type are best vascularized if the contours of the implant are such as to allow a rich proliferation of granulation tissue, the vessels of which appear to be particularly prepared to grow into the implanted bone. The importance of granulation tissue for the success of

bone grafts has been pointed out by Abbott, Schottstaedt, Saunders and Bost (1947), who convincingly explained the superiority in speed of vascularization and incorporation of autogenous cancellous over autogenous cortical bone grafts in terms of graft shape and structure, and granulation tissue formation. Siffert and Barash (1959) have recently shown that bone implanted into a bed of prepared granulation tissue was much more readily invaded by host tissues than was control bone implanted into normal tissues. The extent of host invasion diminished as the granulation tissue matured.

In this experiment it was found that at 14 days the tubular proximal autografts were still significantly less vascular than normal bone, in spite of a nearly normal ratio of short vessels to long vessels which suggested a fully established vasculature in limited areas. At the same time split proximal autografts showed an abnormally high ratio of short to long vessels indicating persisting ingrowth, but they were more evenly vascularized than the tubular grafts. Accepting the view that contact with granulation tissue was the most important factor in the vascularization of these grafts, the observations mentioned above may be explained as follows. In the early stages the tubular implants had contact with granulation tissue in a few shallow concavities on their periosteal surface only. Here contact was achieved quite soon and by 14 days the vasculature was fully established in the corresponding areas in the bone. In split implants the whole of the medullary aspect was eventually bordered by granulation tissue, but because of the depth of this concavity sufficiently close contact for ingrowth of vessels into the grafts occurred later than on the periosteal surfaces of both types of implants. The split grafts therefore were evenly vascularized at 14 days, but the vasculature was not yet fully established throughout.

It thus seems that conditions for vascularization are optimal when the spaces on the implant's surface are wide enough to accommodate a certain amount of granulation tissue, but not so wide as to delay the contact between this tissue and the graft.

Furthermore, from the above arguments it appears likely that autogenous bone implants of the thickness used would be fully vascularized at about 14 days if it were possible to secure sufficiently early contact with granulation tissue on the whole surface of the graft.

Differences in vascularization between autografts and homografts

It has been repeatedly reported (Kiehn *et al.*, 1952 ; Siffert, 1955 ; Stringa, 1957) that bone homografts are less well or less quickly vascularized than bone autografts ; on the other hand, an interference with the graft's blood supply in the course of the homograft reaction has been observed or suggested (Zeiss *et al.* 1958 ; Hammack and Enneking, 1959). The results from the present experiment indicate that both these factors were of significance in the vascularization of homologous implants. It was noted that besides a diminution in the vascularity of homografts from the 6th to the 10th day, ingrowth from the host was slower than in autografts from the very beginning. This was shown by the significant difference in the slope of the regression curves of Class I vessels in the two groups during the first 5 days. It may be that this early reluctance of the host to vascularize homogenous bone was only the first sign of a generally " abnormal " host behaviour, which was later on manifested by the retarded growth and peculiar

features of the new fibrous tissue. Even after 250 days it had often not grown 7·5 mm. into the empty marrow cavity of the tubular homografts, a distance usually covered within 35 days in autografts (Heslop *et al.*, 1960).

From the above observations it would appear that the host can display its ability to react to the presence of foreign tissue at the site of the graft some time before the inflammatory component of the homograft reaction becomes evident. Although most papers on the subject of homografting are mainly concerned with the latter, it is implied by Medawar (1944) and Darcy (1952) that differences in the vascular response to homogenous and autogenous soft tissue grafts exist as early as 4 days. The mechanisms which bring about such an early manifestation of host reactivity at the graft site seem to be unknown and in fact have apparently received little attention.

The behaviour of the granulation tissue round autogenous and homogenous bone implants appeared in large measure to determine the vascularity of the grafts. In the tissue related to most tubular homografts, the late rearrangement of the vascular pattern from a dense capillary network into a sparse system of large vessels seemed to be reflected also in the vasculature of the bone implants. At 35 days for instance the vessels in the tubular homografts were mainly derived from the tissue inside the marrow cavity, and the ingrowth from the periosteal aspect was surprisingly sparse in view of the large number of Class I vessels seen at 14 days after implantation. After 250 days the vascularity of the implants

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EXPLANATION OF PLATES

FIG. 6.—Section of injected proximal normal rat tibia. L.P.

FIG. 7.—Proximal 14-day tubular autograft. Note the patchy vascularization in relation to slight convexities on the outer surface of the graft. L.P.

FIG. 8.—High power view of the subcutaneous ridge of a 14 day autograft. Vessels coming from the outer circumference ramify in the medullary cavity.

FIG. 9.—Fourteen day proximal homograft. The graft is poorly vascularized in spite of ample granulation tissue filling the bays on the outer circumference. L.P.

FIG. 10.—Fourteen day proximal split autograft. The graft is well and evenly vascularized as compared with a tubular autograft. (Fig. 7). L.P.

FIG. 11.—Thirty-five day proximal autograft. The fine capillary network between the trabeculae of new bone in the marrow cavity is seen. The graft is moderately well vascularized. L.P.

FIG. 12.—Lower part of a 35 day proximal homograft. The tissue inside the marrow cavity shows a vascular pattern entirely different from that in autografts (see Fig. 11). The graft is poorly vascularized and its vasculature is mainly derived from the vessels in the marrow cavity. L.P.

FIG. 13.—Two hundred and fifty day proximal autograft. The graft is satisfactorily vascularized; the alteration of its shape by remodelling and the trabeculae in the medullary cavity are well seen. L.P.

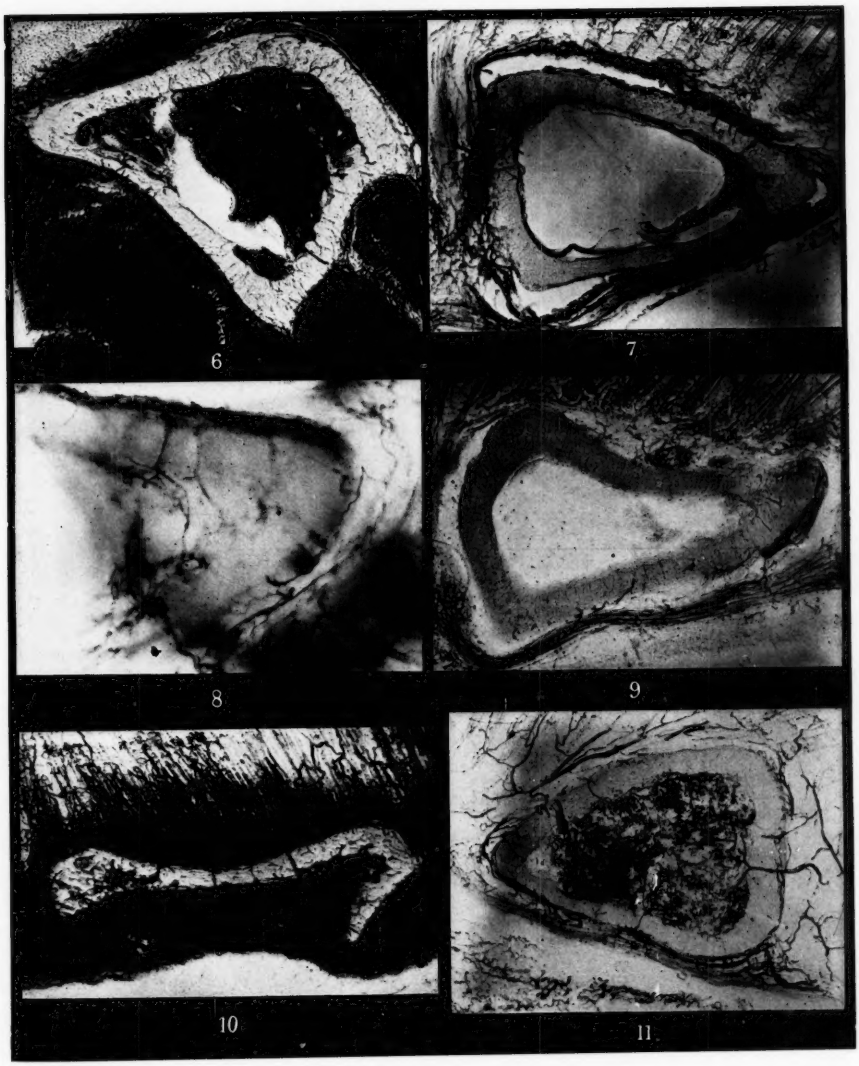
FIG. 14.—Area of 250 day proximal autograft showing the altered vascular pattern related to the remodelling—absorption and replacement—of the graft. Note the capillary system around the osteons in the centre of the field. H.P.

FIG. 15.—Two hundred and fifty day proximal homograft. Note the sparse vascularity of the tissue in the medullary cavity (compare Fig. 12), the relatively meagre vascularity of the graft and the absence of remodelling. L.P.

FIG. 16.—Vessels in a 3 day autograft. The vessels penetrate the whole thickness of the tibial wall and spread in the medullary cavity, suggesting early anastomosis of graft vessels with host vessels and their commencing proliferation. H.P.

FIG. 17.—Vessels in a 3 day homograft suggestive of anastomosis between graft and host vessels at an early stage. H.P.

FIG. 18.—Homologous pinch skin grafts 4 days after transfer to a rat which had received a cortical bone implant from the same donor 10 days prior to skin grafting. The animal was injected intra-arterially with Berlin blue. Note the uninjected halo around the skin grafts, indicating vascular blockage in the course of a secondary response.



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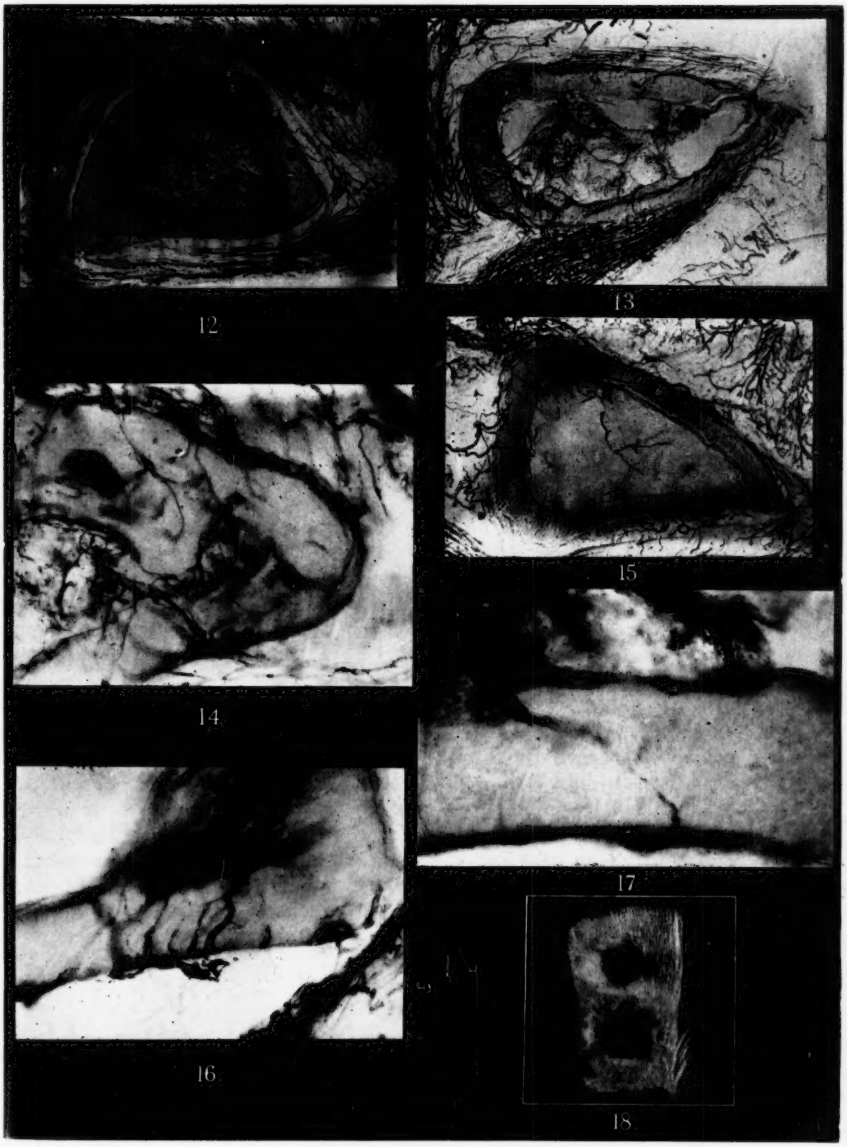

Zeiss, Nisbet and Heslop.

had apparently diminished, as had the vascularity of the host tissue in the marrow cavity.

In autografts, too, vascularization of the implant could always be correlated with the vascular pattern of the granulation tissue or the tissues which came to replace it. Even when new bone and marrow were present in the hollow spaces related to the autografts these areas retained a fine capillary network with numerous connections to the graft's vasculature. These features persisted up to 250 days.

Although the preceding discussion may offer an explanation for the changing vascular pattern peculiar to homogenous grafts, other factors not revealed by the present experiments may have been involved, and it seems in fact likely that this was the case. As the osteocytes of homogenous bone grafts are protected against the immune reaction of the host and consequently survive (Heslop *et al.*, 1960), they most probably act as a continuous source of antigen, thus eventually creating a state of heightened sensitivity. It could well be that in our studies the evidence of this state has been missed, as no biopsies were taken between 14 and 35 days. Such an interpretation would have a strong bearing on the explanation of the vascular features peculiar to homogenous bone grafts reported in this paper. It has been shown that the response to a second graft from the same donor closely resembles the tuberculin reaction and probably is a similar phenomenon (Brent, Brown and Medawar, 1958). As an intense tuberculin reaction will lead to capillary blockage by leucocytes (Gell, 1957), it may be expected that a similar reaction related to a cortical bone graft will interfere with the blood supply of the graft, and might account for the alterations in the vascularity of the homogenous grafts which seem to have taken place between 14 and 35 days. The alterations apparently consisted of a destruction of the vasculature present at 14 days and its replacement by new ingrowth. Indirect evidence for such a suggestion is supplied by the finding that in spite of the large number of vessels entering the grafts from the periphery at 14 days, at 35 days the vasculature of the grafts is mainly derived from a medullary plexus. If the events suggested took place and if they actually were the result of a tuberculin-like reaction, the phenomenon might be analogous to the failure of a highly sensitive host to vascularize a second set skin graft (Billingham, Brent and Medawar, 1954). That blockage of host vessels may occur in the course of a second set reaction in rats is indicated by our own observations. Around skin grafts of 4 days' standing which had been applied 10 days after implantation of cancellous bone grafts from the same homogenous donor, an area about 0·3 cm. deep showed no penetration of the vessels by an intra-arterially injected dye (Fig. 18) (unpublished data).

SUMMARY

The mode, extent and significance of the vascularization of autologous and homologous bone grafts were studied. Grafts of cortical bone from the rat's tibia were implanted suprapannicularly. Several implants were examined on each of the first 14 days, and also at 35 and 250 days. The animals received an intra-arterial injection of Berlin blue and the implants and adjacent tissues were subsequently cleared. The vascularity of the implants was assessed by vessel counts in thick sections. The results were statistically analysed. The data obtained by these methods suggest the following conclusions :

Bone implants of the type used were vascularized mainly by ingrowth of host vessels.

Under favourable conditions some anastomosis of graft and host vessels occurred, but in homografts these vessels were destroyed in the course of the homograft reaction between the 7th and the 12th day.

The most important factor in the vascularization of both types of graft was the presence of granulation tissue, which occurred maximally in the concavities on the surface of the implants, in the empty marrow cavity of the tubular implants and on the concave medullary surface of the split implants and in the large canals of proximal bone.

Significantly slower vascularization of homografts as compared with autografts during the first 5 days after implantation suggested the presence of an antagonism between host and homologous graft at the graft site already at this early stage.

There was a significant difference in the vascularity of homografts and autografts at 14 days, that of the homografts being definitely inferior. This was explicable as a result of antagonism between host and graft resulting in retarded ingrowth and destruction of the anastomosed graft vessels in the course of the homograft reaction.

Autogenous grafts of the type used would probably be completely vascularized by 14 days if it were possible to provide early contact with granulation tissue on all surfaces.

The further development of the vasculature of the implants could be correlated with the eventual vascular pattern of the granulation or its derivatives. In the case of autografts there was extensive development of new bone and marrow with persistence of a fine capillary network which showed many connections with the vessels in the implants. In the case of homografts a sparsely vascular fibrous tissue developed. This was associated with a correspondingly sparse vascularity of the implants.

It seemed possible that in the time between the 14th and 35th day after implantation a second set response may have interfered with the vasculature of the homologous implants.

Distal bone was more slowly vascularized than proximal bone because its compact structure made it less favourable for the development of granulation tissue.

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A CONSIDERATION OF AUTO-ANTIGENS WITH SPECIAL REFERENCE TO HASHIMOTO'S DISEASE

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In two recent communications there have been described, in the serum of individuals with certain diseases, antibodies reacting with antigens contained in the tissue extracts from other individuals but not with extracts of the patient's own tissues. Mackay, Larkin and Burnet (1957) reported this phenomenon with the sera and tissues of cases of disseminated lupus erythematosus, lupoid hepatitis and macroblogulinaemia, using the complement-fixation reaction described by Gajdusek (1957, 1958). Thal, Murray and Egner (1959) have encountered a similar finding in a patient with chronic pancreatitis whose serum precipitated with saline extracts of all human pancreases tested, save the patient's own, whereas sera from other cases of chronic pancreatitis precipitated with the pancreatic extract of the patient in question.

In discussing their findings, Thal *et al.* (1959) consider that the serum factor should be regarded as an iso-antibody, while Burnet (1959) regards the possibility of Gajdusek's complement-fixation reaction being an auto-immune reaction of the Hashimoto type is almost excluded by the failure of the serum factor, in most instances, to react with the autologous antigen. An alternative to these views is the possibility that auto-antibodies are concerned in the above reactions, and that failure to demonstrate the autologous reaction is due to loss of, or modification of, the antigens concerned.

In the present investigation it is shown that the precipitating antibodies to thryoglobulin demonstrable in the serum of many patients with Hashimoto's disease are true auto-antibodies, but that the amount of thyroglobulin extractable from the patient's thyroid gland may be so diminished that it fails to react with any Hashimoto sera, or may contain thyroglobulin which is partially blocked by auto-antibody in such a way that it fails to react with the patient's own serum, although still capable of reacting with certain other Hashimoto sera.

MATERIALS AND METHODS

Thyroid antigens were pepared from thinly sliced frozen thyroid tissue obtained at operation from patients with Hashimoto's disease, thyrotoxicosis, and simple colloid goitre. The slices were added to an equal volume of 0·9 per cent saline solution, extracted for 15 min. in a mechanical shaker at room temperature, and centrifuged at 3000 r.p.m. for 15 min. to remove coarse particles. The extracts and test sera were stored at −15°. In all cases full clinical and laboratory investigations were made before operation and the diagnosis was subsequently confirmed histologically. Extracts of normal thyroids were made as above with material obtained at necropsy.

Precipitin tests were performed by the Ouchterlony technique of double diffusion in agar. Nine cm. diameter Petri dishes containing 20 ml. of 1·7 per cent Difco " Bacto " agar (with

0·1 per cent sodium azide added as preservative) were used. Wells of appropriate size for serum and antigen were cut with a cork borer and diffusion was carried out for 14 days at room temperature.

Electrophoresis of thyroid extracts was carried out on Whatman No. 3 filter paper and on " Oxoid " cellulose acetate membranes. For both media, barbitone buffer of ionic strength 0·1 and pH 8·6 was used and a current of 0·4 mA. per cm. width. Paper electrophoresis was continued for 18 hr., and the fractions were stained with bromophenol blue. Membrane electrophoresis was for 3–4 hr., and the stain used was amido black. Electrophoresis of radioactive thyroid extracts (from patients who had received ^{131}I before thyroidectomy) was carried out on paper and the distribution of radioactivity was determined either by autoradiography (X-ray film being placed in contact with the dried, stained paper strip for 14 days), or by cutting the strip serially into transverse pieces, the radioactivity of which was measured in a well-type scintillation counter. Immuno-electrophoresis of thyroid extracts was performed upon " Oxoid " membranes: following electrophoresis, immune serum, obtained from a patient with Hashimoto's disease, was applied with a pipette along the length of the membrane, which was then kept moist (by immersion in liquid paraffin) for 40 hr. to allow diffusion and precipitation to occur. After removal of the liquid paraffin, free protein was washed out with saline, and precipitation was demonstrated by staining with amido black.

RESULTS

The auto-immune nature of the precipitating antibodies in Hashimoto's disease

Roitt, Campbell and Doniach (1958) have provided convincing evidence that thyroglobulin is the antigenic constituent of thyroid tissue extract which reacts with the precipitating antibodies in the serum of patients with Hashimoto's disease.

Using the Ouchterlony plate technique, we have tested extracts of a large number of normal thyroids with Hashimoto sera, and have failed to demonstrate individual differences in the antigenicity of thyroglobulin, and Witebsky (1957) has described a like result, using Boyden's haemagglutination reaction. If, as these results suggest, there are no thyroglobulin iso-antigens, then it follows either that the precipitating antibodies to thyroglobulin are auto-antibodies, or that Hashimoto's disease develops in individuals whose thyroids are devoid of normal thyroglobulin. Since the condition develops usually in middle-life, after many years of apparently normal thyroid function, the latter alternative seems most unlikely.

Although the indirect evidence outlined above favours the auto-immune nature of the precipitins to thyroglobulin in Hashimoto's disease, there is a striking lack of reports describing autologous precipitin reactions between the serum and thyroid extract of the same patient. We have had opportunity of testing saline extracts of the thyroids of 12 patients whose sera, obtained before thyroidectomy, contained precipitating antibodies to thyroglobulin. Three of the patients (Table, Cases 1–3) had thyrotoxicosis, the occasional association of which with circulating precipitins has been described elsewhere (Goudie, Anderson, Gray, Clark, Murray and McNicol, 1957). The remaining 9 cases had Hashimoto's disease. In 6 cases (1–6) the autoimmune nature of the antibodies was established in precipitin tests with the patient's own thyroid extract. The sera of these 6 cases were found to react also with extracts of thyroids of other individuals. The thyroid extracts of Cases 3–6 were of low antigenic potency and were unsuited to more detailed investigation. Cases 1–3 provided more potent antigenic extracts, and the sera of these 3 cases were observed to react in identical fashion with the autologous

and with other thyroid extracts: this observation was made by performing precipitin tests on Ouchterlony plates, the thyroid extracts being placed in wells arranged in a ring around a central well containing the serum (Fig. 2). Wilson and Pringle (1956) have shown that, with such an arrangement of antigen and antibody wells, a precipitating antibody will distinguish readily between the corresponding and closely related antigens by the formation of spurs of precipitate. In the present investigation, the serum of Case 1 was tested with the autologous and 20 other thyroid extracts, including extracts of 8 normal thyroids; the sera of Cases 2 and 3 were tested with the autologous and 4 other extracts. In all tests, the adjacent precipitates fused completely without spur formation, a result which suggests that Cases 1–3 have antigenically normal thyroglobulin, and that auto-immunization has not developed as a result of loss or acquisition of an antigenic determinant group in the thyroglobulin molecule.

Loss of auto-antigen in Hashimoto's disease

Histological examination of the thyroid gland in Hashimoto's disease shows the colloid to be invariably and grossly diminished (Fig. 1). Accordingly, saline extracts of Hashimoto thyroids are likely to be poor in thyroglobulin, and in fact electrophoresis of such extracts invariably shows diminution in the staining intensity of the thyroglobulin band (Fig. 6). Since thyroglobulin is the antigen participating in the precipitin reaction, Hashimoto thyroid extracts would be expected to be of low antigenic potency, and this was found to be so for the 9 Hashimoto extracts investigated. Extracts from Cases 4–6 (Table) provided

TABLE.—*Precipitin Tests upon the Thyroid Extracts of Patients with Precipitating Antithyroglobulin in the Serum*

Case No.	Diagnosis	Results of precipitin tests with: Patent's own serum	Results of precipitin tests with: Other precipitating serum
1–3	Thyrotoxicosis	+	+
4–6	Hashimoto	+	+
7–11	Hashimoto	−	−
12	Hashimoto	−	+

antigens of low potency, and those from Cases 7–11 failed to form a visible precipitate when tested with the autologous and other precipitating sera.

Modification of auto-antigen in Hashimoto's disease

Paper electrophoresis, followed by auto-radiography of the electrophoretic strip, was performed upon the thyroid extracts of patients who had received 100–150 μc of ^{131}I 24–48 hr. before thyroidectomy. Fig. 5 shows the results with two thyroid extracts, one from a patient with thyrotoxicosis whose serum did not contain precipitating antibody to thyroglobulin, and the other from a patient with Hashimoto's disease (Case 4). The radioactivity of the thyrotoxic extract corresponds to the electrophoretic mobility of normal thyroglobulin, whereas Case 4 extract shows radioactivity extending from behind the origin to the zone of migration of normal thyroglobulin. A similar result was obtained with two other

Hashimoto extracts investigated by this technique, and is regarded as indicating either that the metabolism of the thyroid is altered in such a way that the radio-iodine has been incorporated into substances other than thyroglobulin, or that the thyroglobulin has been modified by union with auto-antibody. Evidence favouring the latter alternative was provided by immuno-electrophoresis of Hashimoto thyroid extracts. This procedure presents technical difficulties owing to the low antigenic potency of such extracts, but in some cases the antigen was demonstrable by applying a precipitating (Hashimoto) serum directly on, instead of alongside, the electrophoretic run. When a precipitate was demonstrable, it was always closer to the origin than that formed by immuno-electrophoresis of a normal thyroid extract (Fig. 7). It thus appears that the slow and heterogeneous migration of ^{131}I in radioactive Hashimoto thyroid extracts is attributable, at least in part, to the diminished migration rate of a substance identified immunologically as thyroglobulin. Before concluding that the altered electrophoretic mobility of the thyroglobulin is attributable to union with auto-antigen, it is necessary to produce evidence, firstly that the Hashimoto thyroid extract may contain auto-antibody, and secondly that union with antibody can result in diminution of the electrophoretic mobility of thyroglobulin. The first requirement was fulfilled in a startling fashion by precipitin tests upon the thyroid extract of Case 11, which behaved not as an antigen, but as an antibody, reacting with normal thyroid extracts. The second requirement was fulfilled by demonstrating that admixture of a radioactive thyroid extract with a Hashimoto serum resulted in diminished electrophoretic mobility of radioactivity, whereas admixture with normal serum had no such effect (Fig. 8).

It is concluded that the thyroglobulin present in Hashimoto thyroid extract is modified by union with auto-antibody, either *in vivo*, or by admixture with the blood contained in the thyroid, during preparation of the extract. Such modification doubtless contributes to the failure, in some instances, to demonstrate immunologically even a reduced amount of thyroglobulin in the thyroid extract.

Iso-precipitation without auto-precipitation

Case 12 was unique in that the patient's serum contained precipitin demonstrable with other thyroid extracts but not with the patient's own, while the patient's thyroid extract gave a positive reaction when tested with another Hashimoto serum (serum L). In view of the findings described in the preceding section, this result suggested that the thyroglobulin in Case 12 thyroid extract was partially blocked by auto-antibody in such a manner that it was no longer precipitable by Case 12 serum, and yet still contained antigenic receptors available for precipitation by serum L. This explanation is supported by the following two experiments. Firstly, it was shown that a mixture of equal volumes of Case 12 serum and a 1 in 16 dilution of a normal thyroid extract provided an antigen which precipitated, in agar diffusion tests, with serum L, but not with Case 12 serum (Fig. 3). In other words, the serum of Case 12 is capable of modifying a thyroid extract in such a manner that it loses its antigenicity for Case 12 serum while retaining antigenicity for serum L. Secondly, in precipitin tests in which Case 12 serum and serum L were placed in wells equidistant from a third well containing normal thyroid extract, the line of precipitate formed by serum L did not fuse completely with, but rather extended beyond, that formed by Case 12 serum (Fig. 4). This suggests that there is more than one type of antigenic determinant

in thyroglobulin, and that an antibody is present in serum L, but not in Case 12 serum, which reacts with one particular thyroglobulin antigenic determinant.

DISCUSSION

It has been demonstrated convincingly by Roitt *et al.* (1958) that the precipitating antibodies associated with Hashimoto's disease react specifically with thyroglobulin, and it follows that they should react with the patient's own thyroglobulin, *i.e.* as auto-antibodies. The direct confirmation that in some cases (Table, Cases 1–6) this is so requires no further comment. Of greater interest is the failure, in Cases 7–12, to demonstrate the autologous reaction. Clearly this is due, in Cases 7–11, to non-antigenicity of the thyroid extracts, for these failed also to react with precipitating sera from other individuals. The explanation advanced for the loss of antigenicity of these thyroid extracts is twofold. Firstly, the Hashimoto thyroid gland is poor in colloid and hence in thyroglobulin, and secondly, some or all of the thyroglobulin present in such extracts is united with auto-antibody to form soluble complexes. Roitt *et al.* (1958) have demonstrated such complexes in mixtures of purified thyroglobulin and certain Hashimoto sera, and Goudie, Anderson and Gray (1959) have shown that such complex formation may protect the thyroglobulin from precipitation by precipitating sera.

The results with Case 12 are of particular interest, for they provide an example of failure to demonstrate the autologous reaction in a patient whose serum contains precipitating antibody which reacts with thyroid extracts from other individuals, and whose thyroid extract is antigenic for other precipitating sera. The explanation advanced for this result, namely that the thyroglobulin in Case 12 thyroid extract is partially blocked by auto-antibody, is at first sight difficult to accept, for it might appear that such blocking would prevent precipitation not

EXPLANATION OF PLATES

FIG. 1.—Comparison of the colloid content of normal (*a*) and Hashimoto (*b*) thyroids. The colloid appears black. PAS ×19.

FIG. 2.—" Reaction of identity " between autologous (top and bottom wells) and isologous thyroid extracts. The central well contains the serum of Case 1.

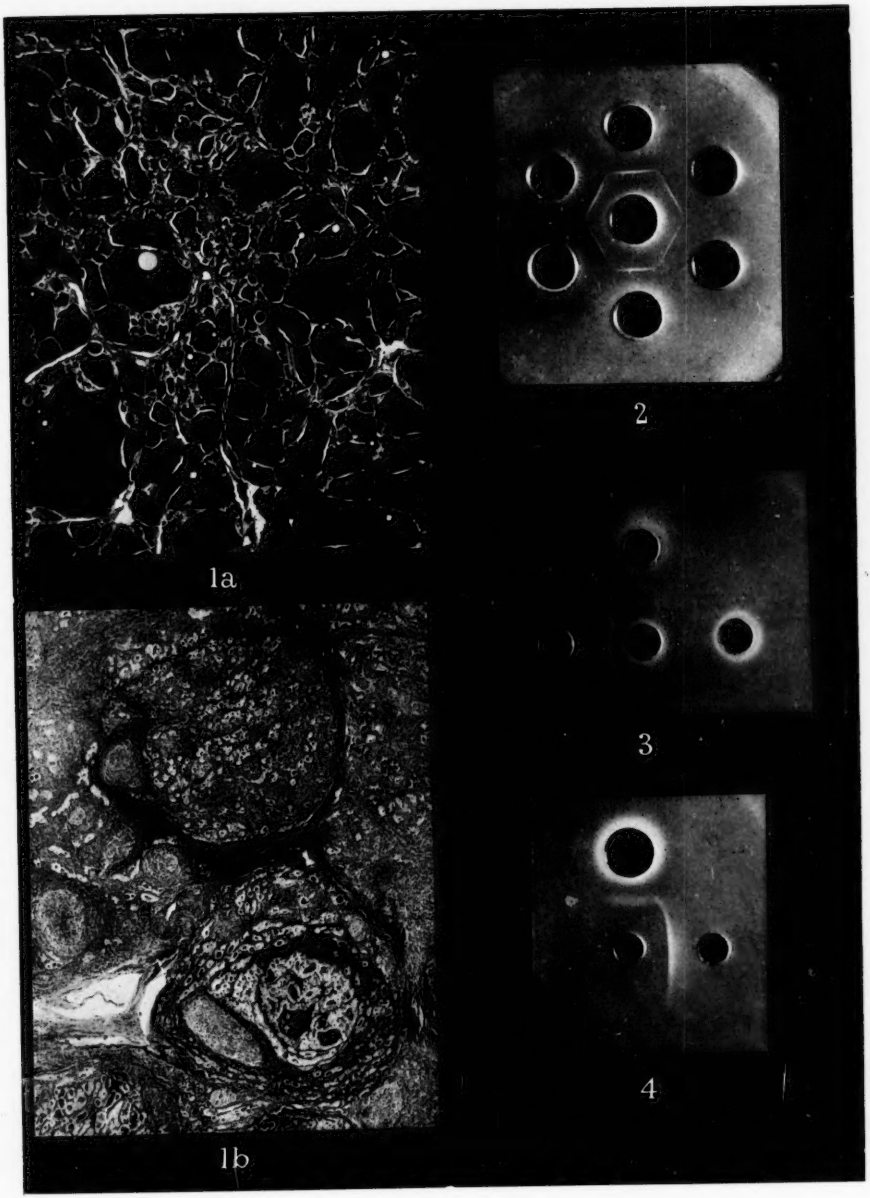
FIG. 3.—" Iso "-precipitation without auto-precipitation. The antigen (central well) is normal thyroid extract mixed with serum of Case 12. Top well, normal serum ; right well, serum of Case 12 ; left well, serum L. Only this last serum has reacted to give a precipitate.

FIG. 4.—The non-identity of precipitating antibodies of the serum of Case 12 (top well) and serum L (right well). The third well contains normal thyroid extract. The precipitate formed by the serum of Case 12 is crossed by that formed by serum L.

FIG. 5.—Autoradiographs of electrophoretic strips of radioactive thyroid extracts showing the normal pattern and the appearances in Hashimoto's disease (Case 4), in which there is retarded migration or trailing of radioactive constituents.

FIG. 6.—Electrophoresis of normal (left) and Hashimoto (right) thyroid extracts. The thyroglobulin band is greatly diminished in the Hashimoto extract.

FIG. 7.—Immuno-electrophoresis of normal (left) and Hashimoto (right) thyroid extracts. The outer part of each run was cut off and stained after electrophoretic separation : the serum of Case 6 was applied along the central part of each run for immuno-electrophoresis. The rather faint precipitate formed with the Hashimoto thyroid extract is nearer to the origin than is the more intense precipitate formed with thyroglobulin of the normal thyroid extract.



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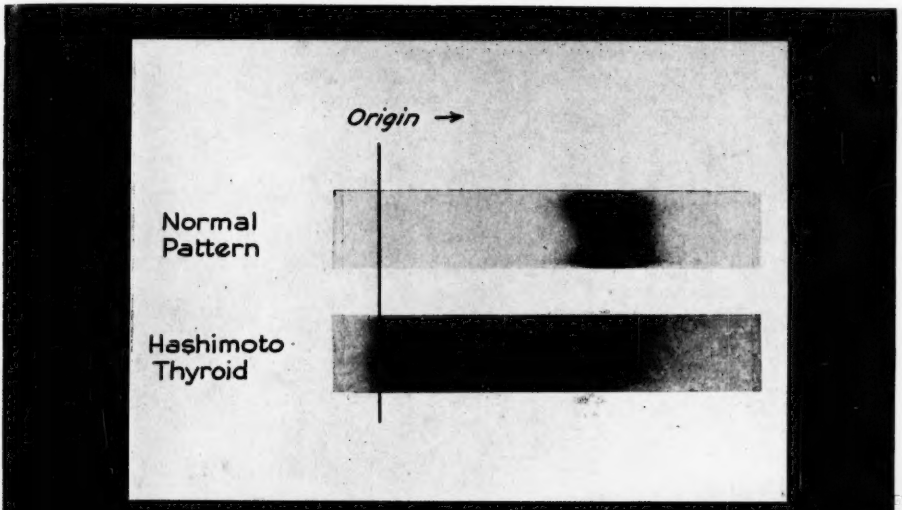

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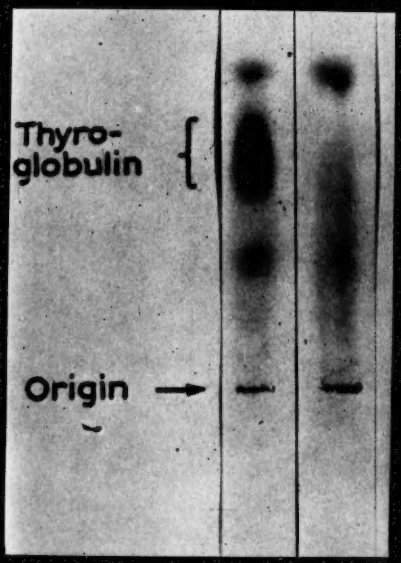

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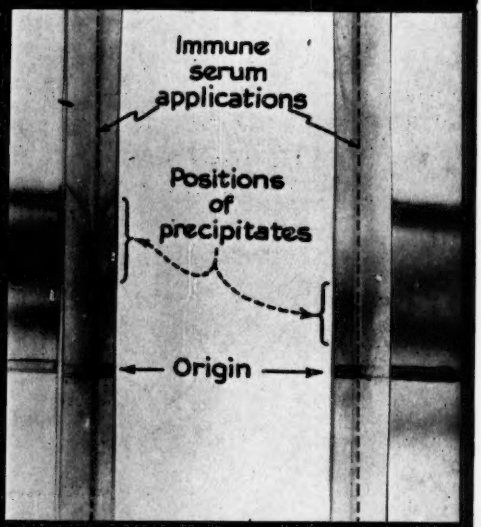

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Anderson, Goudie and Gray.

only by the autologous, but also by other precipitating sera. There are at least two possible explanations of why this is not invariably so. Firstly, thyroglobulin molecules may differ in antigenicity, as has been suggested by Roitt *et al.* (1958), in which case the serum of Case 12 might react with, and annul, molecules of a

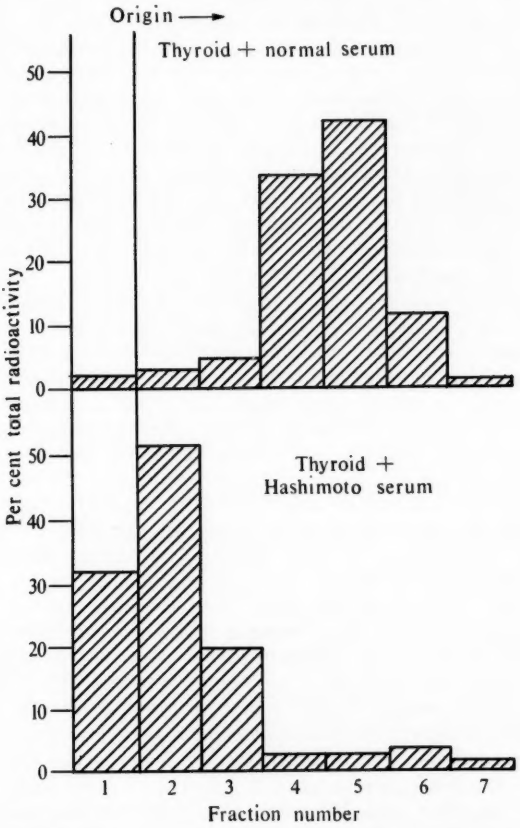

FIG. 8.—Distribution of radioactivity in fractions of paper electrophoretic strips of thyrotoxic thyroid extract mixed with normal serum (above) and Hashimoto serum (below). The strips were cut into transverse pieces and radioactivity was measured in a well type scintillation counter. The migration of thyroglobulin mixed with Hashimoto serum is similar to that observed for radioactive Hashimoto thyroid extract.

certain antigenic specificity, leaving others of a different specificity unaffected and available to react with serum L. A second possibility is that all thyroglobulin molecules are antigenically identical, and that each molecule contains two or more antigenically different determinant groups. Evidence for this is provided in a recent report (Goudie *et al.*, 1959). If this is correct, then the results with Case 12 are explicable on the assumption that the thyroglobulin of Case 12 thyroid extract

has one particular receptor, say X, blocked by non-precipitating auto-antibody, leaving a second receptor, say Y, available to react with the corresponding antibody present in serum L but not in Case 12 serum.

While it remains uncertain which, if either, of the above two possibilities is correct, the evidence is strong that the failure of the autologous reaction for Case 12 is attributable to partial blocking of the thyroglobulin by auto-antibody, for admixture with Case 12 serum was found to confer upon a normal thyroid extract the antigenic properties of Case 12 extract. Partial blocking of a tissue antigen by auto-antibody might conceivably occur with other antigen-antibody systems. Thus the failure reported by Thal *et al.* (1959) to demonstrate the autologous precipitin reaction for a patient with chronic pancreatitis whose serum precipitated with pancreatic extracts from other individuals, does not seem to justify the antibody being regarded as an iso-antibody, as these authors have done. Similarly, failure in most instances to demonstrate directly the autologous nature of Gajdusek's complement-fixation reaction does not appear to justify Burnet's (1959) conclusion that the possibility of its being an auto-immune reaction of the Hashimoto type is almost excluded. Roitt *et al.* (1958) have estimated that thyroglobulin has a valency of only 4 for the antibodies in Hashimoto sera. If this is so, the antigenic complexity is limited, and this may explain why we have encountered only one case in which " iso-antigenicity " but not auto-antigenicity was demonstrable. In Gajdusek's complement-fixation reaction, in which iso-antigenicity but not auto-antigenicity is the usual finding, the antigenic material consists of relatively large particles (Mackay and Gajdusek, 1958), permitting the possibility of a much greater complexity of antigenic determinants. In fact, in this reaction the concept that auto-antibodies may react with a small proportion of the antigenic determinant groups, and thereby block further auto-antibody union, provides a possible explanation for the " inhibitor of unbelievable specificity " referred to by Mackay *et al.* (1957).

In recent years, some confusion has arisen over the use of the terms auto- and iso- as prefixes to antibodies reacting with tissue constituents. While we consider that the proof of auto-immunization requires the demonstration that the antibodies concerned react with a normal constituent of the patient's own tissues, the present investigation shows that, in many instances, this may be difficult or impossible owing to loss of the antigenic constituent concerned, or its being blocked by auto-antibody. In other instances, such as the complement-fixation reaction between adrenal extracts and the serum of patients with Addison's disease (Anderson, Goudie, Gray and Timbury, 1957) the autologous nature of the reaction cannot be tested as the patient's tissue is not normally excised. In this situation, it seems unjustifiable to regard the antibodies as iso-antibodies as Cruickshank, Currie and Symington (1957) have advocated.

It is clear, from the present investigation, that failure to demonstrate the autologous reaction does not exclude the possibility that auto-immunization has occurred. When the serum of a patient contains a globulin factor which can be shown, by an acceptable immunological technique, to react invariably and specifically with the extract of a particular non-diseased organ, or a tissue constituent, from a large number of individuals, and when the presence of the serum factor is associated with pathological changes in the particular organ or tissue with which it reacts, then auto-immunization, and not iso-immunization, should be presumed to have occurred.

SUMMARY

Extracts of thyroids obtained surgically from 12 patients whose sera contained precipitating antithyroglobulin were examined for antigenicity. The autologous precipitin reaction was demonstrated directly in 6 cases. No antigen was demonstrable in 5 extracts using the patient's own and other precipitating sera, and evidence is presented that the failure of such Hashimoto thyroid extracts to behave as antigen is due to marked diminution of antigen (thyroglobulin) content, probably with, in addition, blocking of the remaining antigen by auto-antibody. In one case no precipitate formed between the thyroid extract and the patient's own serum, but another Hashimoto serum formed precipitate with this extract: this phenomenon is shown to be attributable to blocking of auto-antigen by auto-antibody and the seemingly isologous reaction is due to a difference in specificity of the auto- and " iso "-antibodies concerned.

These findings show how auto-antibodies may be mistakenly regarded as iso-antibodies. The principles concerned may be applicable to other systems in which the antibodies apparently fail to react with auto-antigens.

We wish to thank Dr. E. M. McGirr of the University Department of Medicine at the Royal Infirmary, Glasgow, who kindly supplied some of the sera and thyroid extracts used in this investigation.

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THE MECHANISM OF GLYCOGENOLYTIC ACTION OF ENDOTOXIN

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DEPLETION of liver glycogen and hyperglycaemia as a result of endotoxin poisoning is a well documented phenomenon (Menten and Manning, 1924 ; Zeckwer and Goodell, 1925 ; Tal and Olitzki, 1948 ; Thomas, 1954). However, little is known about the mechanism causing these disturbances. Kun and Miller (1948) suggested that the breakdown of glycogen and the quantitative changes in blood glucose, lactate and pyruvate were due to tissue anoxia, resulting from the inhibition of succinic oxidase by endotoxin. In view of the fact that crude preparations of the bacterial endotoxin were used in previous work on these effects, this study was undertaken in order to examine the mechanism underlying the changes in carbohydrate metabolism due to a purified preparation of bacterial lipopolysaccharide.

MATERIALS AND METHODS

Preparation of endotoxin

A strain of *Chromobacterium prodigiosum* was grown in 25 l. of liquid medium. The cells were harvested by centrifugation in the Sharples centrifuge and washed twice by resuspending in large quantities of distilled water. The packed cells were taken up in about 200–300 ml. of distilled water, poured into 10 volumes of acetone chilled to $-10°$ and collected by centrifugation in a refrigerated centrifuge at $-5°$. After 2 additional washings with acetone, the bacteria were quickly dried in vacuum. The yield of acetone-dried bacteria was 1 g./l. of culture medium

The endotoxin was purified by a modification of the method of Westphal and Luderitz (1954). The acetone-dried cells were extracted with 75 per cent phenol. After dialysis against water to remove the phenol, the endotoxin was precipitated by the addition of 6 volumes of absolute alcohol. All the procedures were carried out in the cold (4°). The alcohol precipitate was dissolved in water, the alcohol was removed by dialysis and the preparation was lyophilized. The rat MLD of this preparation of endotoxin was 0·4 mg./100 g. body weight.

The experiments were carried out on male albino rats of the local strain weighing 100–120 g. Endotoxin was administered intraperitoneally, each rat receiving 2 mg. toxin dissolved in 0·5 ml. saline. Control animals were given the same amount of saline by intraperitoneal injection. The animals were killed under sodium pentobarbital anaesthesia, bled by cutting the abdominal blood vessels and the liver removed and immersed in cold saline. Liver slices about 0·5 mm. thick were cut with the Stadie and Riggs tissue slicer (1944). Livers were homogenized in 0·154 M KCl in the cold by the aid of a Teflon homogenizer. Purified rabbit liver phosphorylase was prepared according to Sutherland and Wosilait (1955) up to the 4th step of the preparation procedure.

Glycogen was precipitated by the method of Good, Kramer and Somogyi (1933) and determined colorimetrically with anthrone reagent. Inorganic phosphate was determined by a modification of the method of Fiske and Subbarow (1925).

RESULTS

The glycogen content of the diaphragm dropped sharply in the first hour following the administration of endotoxin. In the liver such a decrease could be demonstrated only after 2 hr. By the end of 4 hr. the glycogen reserve of both the liver and the diaphragm was almost completely depleted. In adrenalectomized rats, liver glycogenolysis was increased by toxin administration. Muscle

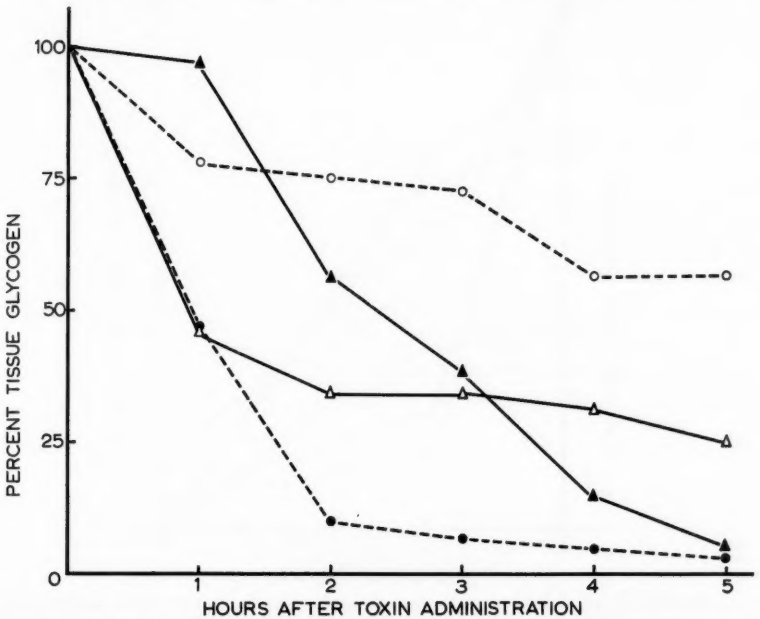

FIG. 1.—Liver and diaphragm glycogen after endotoxin administration to normal and adrenalectomized rats.

▲ Normal rats—liver glycogen.
△ Normal rats—diaphragm glycogen.
● Adrenalectomized rats—liver glycogen.
○ Adrenalectomized rats—diaphragm glycogen.

carbohydrate metabolism, on the other hand, was affected by toxin treatment to a lesser extent in the adrenalectomized than in unoperated animals (Fig. 1). The glycogenolytic effect of the injected endotoxin can be demonstrated also *in vitro* (Fig. 2). Liver slices prepared from toxin treated normal and adrenalectomized animals (2 hr. after toxin administration) showed increased glycogenolysis when compared to normal and adrenalectomized controls. Glycogen synthesis from glucose was markedly impaired in liver slices of normal and adrenalectomized toxin treated rats when compared to the respective controls.

Homogenates prepared from livers of toxin treated rats were more active in the phosphorolytic breakdown of glycogen than those from normal livers. This

increase was measurable only when more than 2 hr. had elapsed after the administration of the toxin (Fig. 3).

From these experiments it was concluded that one of the enzymes participating in the phosphorolytic breakdown of glycogen is stimulated by the administration of endotoxin. Since liver phosphorylase is known to be the rate-limiting enzyme in the breakdown of glycogen (Sutherland and Cori, 1951) it was the first to be

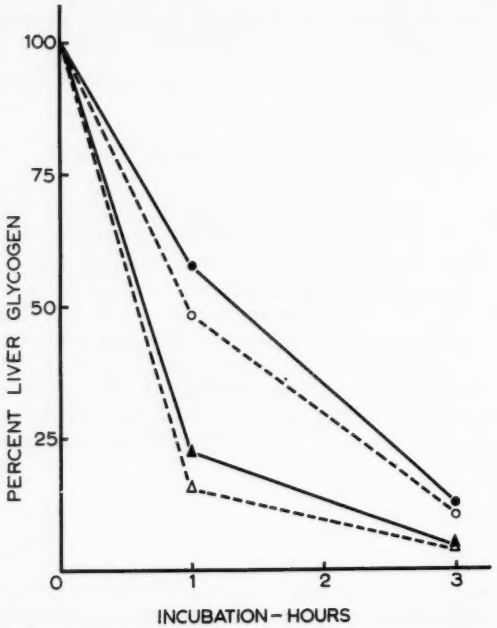

FIG. 2.—Glycogenolysis in liver slices of normal and adrenalectomized toxin treated rats.

▲ Normal toxin treated.
● Normal control.
△ Adrenalectomized toxin treated.
○ Adrenalectomized control.

Liver slices were incubated at 37° with shaking in 1 ml. of a slightly modified Krebs-Ringer phosphate medium of the following composition : NaCl 120 mM, KCl 4·8 mM, $CaCl_2$ 2·6 mM, $MgSO_4.7H_2O$ 1·2 mM, Na-phosphate 16·6 mM, $NaHCO_3$ 16·6 mM pH 7·4. The bottles were gassed 1 min. before incubation with a mixture of 95 per cent O_2–5 per cent CO_2.

tested. It could be shown that endotoxin administration markedly increased phosphorylase activity, as measured by the conversion of glucose-1-phosphate into glycogen (Fig. 4).

In order to test whether the higher phosphorylase activity was due to an increase in the amount of the enzyme or to its activation, the following experiment was performed :

Liver homogenates obtained from control and toxin treated rats were preincubated in citrate buffer for time intervals ranging between 5 and 30 min. In parallel experiments NaF or adenylic acid was added to the reaction mixture as

both are known to inhibit the inactivation of phosphorylase by the liver homogenate (Wosilait and Sutherland, 1956). At the end of the pre-incubation the phosphorylase activity was determined by adding the phosphorylase assay system and incubating for 10 min. (Fig. 5).

It was found that in the beginning there was a considerable difference in the phosphorylase activity of liver homogenate of normal and toxin treated rats.

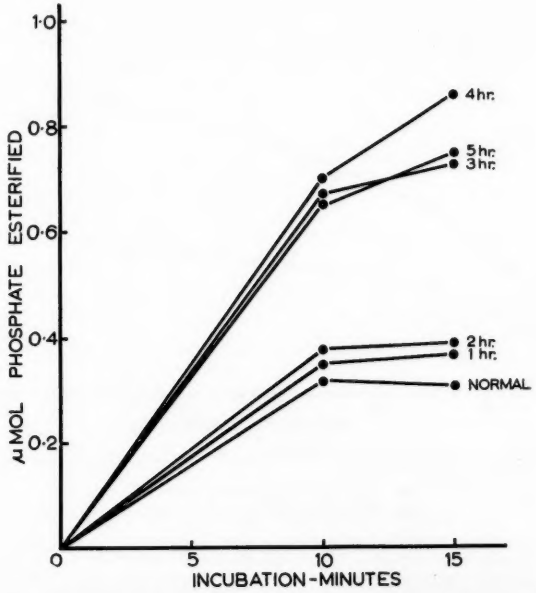

FIG. 3.—Glycogen phosphorolysis by liver homogenates of toxin treated rats at different time intervals after toxin administration. 0·4 ml. of a liver homogenate prepared in 4 vol. of isotonic KCl (see methods) were added to 1·1 ml. Krebs-Ringer phosphate medium of the following composition: KCl 1·15 per cent 4 ml., $MgSO_4$ 0·8 per cent 1 ml., NaCl 0·85 per cent 100 ml., phosphate buffer 0·01 M 15 ml., NaF M/40, glycogen 10 per cent, pH—7·4. Incubation was carried out at 37° under nitrogen. The reaction was stopped by adding 2·5 ml. 10 per cent trichloroacetic acid.

However, pre-incubation for 5 min. in the presence of adenylic acid was enough to bring the phosphorylase activity in the two preparations to the same level. There did not seem to be an increase in the absolute amount of enzyme following toxin administration. In toxin treated rats, all the liver phosphorylase was in the active state, whereas in normal rats only 60 per cent of the enzyme was in this form.

This conclusion could be corroborated by the finding (Table I) that the phosphorylase activity of homogenates from toxin treated rats could not be increased by the administration of adrenaline. This substance has been shown by Sutherland and Cori (1951) to bring about full activation of liver phosphorylase. On the other hand, marked phosphorylase activation was obtained by adrenaline administration

to normal rats. Homogenates of toxin treated and adrenaline treated rats showed equal enzyme activity.

In order to differentiate between two possible alternatives of phosphorylase activation by endotoxin, *i.e.* increased reactivation of the enzyme or inhibition

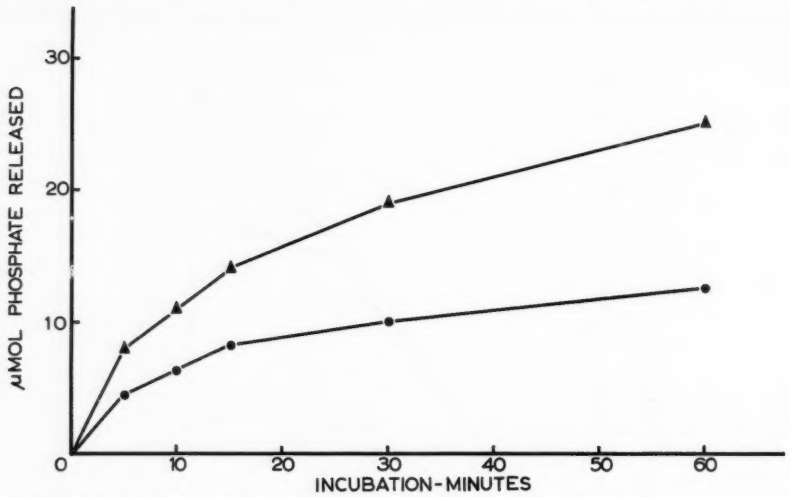

FIG. 4.—Phosphorylase activity in toxin treated and normal liver homogenates.

● Normal liver homogenate.
▲ Toxin treated liver homogenate.

0·4 ml. of a 5 per cent w/v liver homogenate were added to 1·1 ml. of the " phosphorylase assay system " made up as follows : 15 μmoles glucose-1-phosphate, 50 μmoles NaF, 7·5 mg. glycogen, 50 μmoles citrate buffer pH 6. The mixture was incubated for the indicated time intervals at 37° in air. Reaction was stopped by addition of 2·5 ml. of 10 per cent trichloroacetic acid.

TABLE I.—*Adrenaline Effect on Phosphorylase Activity in Normal and Toxin Treated Liver Homogenate*

Tissue preparation	Number of experiments	μmole phosphate liberated
Normal rat liver homogenate . . .	5	3·84±0·12
Ditto—treated with adrenaline . .	5	5·30±0·63
Toxin treated rat liver homogenate .	5	5·26±0·20
Ditto—treated with adrenaline . .	5	5·46±0·20

Conditions of phosphorylase assay as in Fig. 4 except for concentration of the homogenate, which was 25 per cent w/v in isotonic KCl.

of its inactivation, purified liver phosphorylase was prepared and used as substrate and liver homogenates of toxin treated and normal rats were used as inactivating and reactivating enzymes. No change was found in the rate of inactivation of purified liver phosphorylase by toxin treatment (Fig. 6).

After partial inactivation of purified liver phosphorylase by incubation for 20 min. with homogenate of normal and toxin treated liver, the enzyme could be reactivated by the addition of ATP and Mg^{++} and incubation for a further

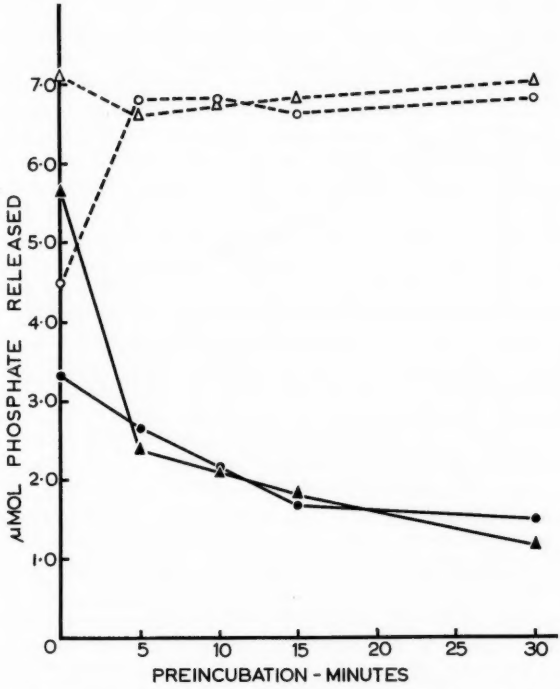

FIG. 5.—Effect of pre-incubation on phosphorylase activity or normal and toxin treated liver homogenates.

▲ Toxin treated liver homogenate.
△ Toxin treated liver homogenate + AMP.
● Normal liver homogenate.
○ Normal liver homogenate + AMP.

0·4 ml. of 25 per cent liver homogenate were incubated for different time intervals in 50 μmoles citrate buffer with and without 5 μmoles AMP. At the end of the pre-incubation period phosphorylase assay system was added and incubation carried out as described in Fig. 4.

10 min. An aliquot of the reaction mixture was transferred to the phosphorylase assay system (Fig. 4) and the extent of reactivation was measured (Table II). It can be seen that the activating enzyme phosphorylase kinase (Rall, Sutherland and Wosilait, 1956) is more active in homogenates of toxin treated livers.

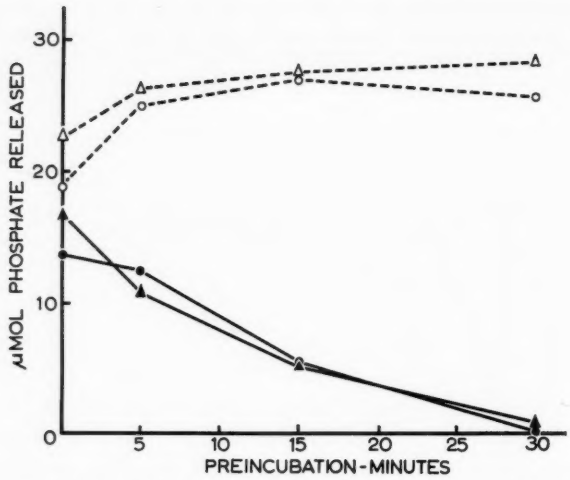

FIG. 6.—Inactivation of purified liver phosphorylase by normal and toxin treated liver homogenates.

● Normal liver homogenate.
○ Normal liver homogenate + AMP.
▲ Toxin treated liver homogenate.
△ Toxin treated liver homogenate + AMP.

Purified rabbit liver phosphorylase was incubated with 0·4 ml. of 1 per cent liver homogenates from normal and toxin treated rats in 50 μmoles citrate buffer with and without 5 μmoles AMP.At the end of the pre-incubation time the phosphorylase assay system was added and the phosphorylase activity was determined after an additional incubation of 10 min.

TABLE II.—*Reaction of Inactivated Liver Phosphorylase by Normal and Toxin Treated Liver Homogenate*

Tissue preparation	Number of experiments	Phosphorylase activity after inactivation	Extent of reactivation
Normal rat liver homogenate	9	0·81±0·07	0·39±0·04
Toxin treated rat liver homogenate	9	0·79±0·11	0·72±0·08

For experimental details see text. The results are expressed as μmole phosphate released in the presence of 0·4 ml. 1 per cent liver homogenate. Initial phosphorylase activity added before inactivation was 3·3 μmole P.

DISCUSSION

Glycogen depletion and hyperglycaemia which are characteristic symptoms of endotoxin poisoning are shown to be the result of increased phosphorylase activity in the liver. This increased activity after toxin administration could be demonstrated *in vitro* in isolated liver preparations. Slices and homogenates also showed increased glycogenolysis and markedly impaired ability to synthesize glycogen.

According to Sutherland and Cori (1951), the concentration of active phosphorylase in the liver represent the result of a balance between two opposite reactions. the inactivation of the enzyme by a specific phosphatase and its reactivation by a second enzyme in the presence of ATP and Mg^{++}. The enzymatic inactivation of phosphorylase proceeds with the release of inorganic phosphate, while the reactivation of the dephospho-phosphorylase proceeds with the transfer of phosphate to the enzyme protein (Sutherland and Wosilait, 1955 ; Rall *et al.*, 1956).

Our results showed that phosphorylase of toxin treated liver was not more stable to *in vitro* inactivation than was the normal liver phosphorylase, nor was the phosphorylase-phosphatase activity diminished after endotoxin administration. Decreased inactivation of phosphorylase as the cause of higher phosphorylase activity was thus excluded. The higher phosphorylase-kinase activity, found in toxin treated rat liver, seems to be the underlying cause of maximal phosphorylase activity after endotoxin administration.

Sutherland and his co-workers showed that phosphorylase-kinase is activated *in vivo* and in tissue preparations by addition of adrenaline and glucagon (Sutherland and Cori, 1951 ; Rall *et al.*, 1956). The similarity of the mechanism of phosphorylase activation by endotoxin or adrenaline administration raises the question of the role of adrenaline secretion in endotoxin poisoning. Although many investigators (Delaunay, Delaunay and Lebrun, 1947 ; Tal and Olitzki, 1948 ; Boquet and Izard, 1950 ; Boquet, 1951) seem to agree that a great number of physiological and pathological changes accompanying endotoxin poisoning are similar to those seen after adrenaline administration, positive proof of increased adrenaline secretion is scarce (Armin and Grant, 1957 ; Scherr and Glaviano, 1959 ; Mundy and Mehlman, 1959). Our findings point to the conclusion that the adrenal gland plays no important part in the mechanism underlying the changes in carbohydrate metabolism in the liver since the above discussed enzymatic changes could also be found in adrenalectomized animals. It may be that endotoxin causes a sensitization to adrenaline, bringing about a greatly augmented response to normal amounts of adrenaline (Zweifach, Nagler and Thomas, 1956). The changes in carbohydrate metabolism of muscle showed a higher degree of dependence on the adrenal gland. The finding that the glycogenolytic effect can be demonstrated only very late, after many other manifestations of intoxication are present, suggests that the activation of phosphorylase is not the result of a direct action of endotoxin, but occurs at a later time as a result of previous changes in the general metabolism.

SUMMARY

The accelerating effects of endotoxin injection on glycogenolysis in muscle and liver could also be demonstrated with slices and homogenates of toxin treated rats.

The increased glycogenolysis in the liver in response to endotoxin persisted after adrenalectomy, while that in the muscle was markedly reduced.

Increased liver phosphorylase activity was found to account for the increased glycogenolysis.

Toxin treatment converted practically the entire liver phosphorylase to the active form.

Evidence is submitted to show that the total activation of liver phosphorylase by endotoxin is brought about by increased activity of the phosphorylase-kinase. The relation of this endotoxin effect to adrenaline is discussed.

We wish to thank Prof. J. Mager for his interest and help during various phases of this work.

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EXPERIMENTAL PYELONEPHRITIS: REACTIVATION OF THE HEALING LESION BY URETERIC OCCLUSION

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EXPERIMENTAL pyelonephritis can be produced in rabbits if the ureter is occluded and *Bacterium coli* injected intravenously. If the occlusion is removed, the inflammatory process gradually subsides leaving a kidney whose macroscopic and microscopic appearances are similar to those found in human chronic pyelonephritis (Mallory, Crane and Edwards, 1940; Heptinstall and Gorrill, 1955). In rats, a temporary occlusion of the ureter for 24 hr. combined with intravenous injection of *Bact. coli* causes an infection which, based on whole kidney organism counts, increases up to the 4th day when abscesses are present, and then undergoes a progressive decrease until at 4 weeks the kidneys show low counts (Brumfitt and Heptinstall, 1958). Failure to release the ureteric obstruction leads to progressive infection of the kidney until the parenchyma is destroyed. Such experiments show that even an established infection requires continued ureteric obstruction if it is to progress. Because of the effects of release of obstruction on the healing of infection it was decided to test the reverse effect and see if infection could be reactivated in the healing pyelonephritic kidney by occlusion of the ureter.

MATERIALS AND METHODS

Both ureters of female albino Wistar rats (Glaxo), weighing 180–200 g., were obstructed and after 30–45 mins. a culture of *Bact. coli* was injected into the jugular vein. The obstruction to both ureters was released after 24 hr. The strain and dose of organism, the method of producing temporary ureteric occlusion and other bacteriological procedures were the same as previously described (Brumfitt and Heptinstall, 1958). The rats were then divided into 2 groups, 18 in the first and 14 in the second. In the first group the left ureters of 10 of the animals were completely occluded by a silk ligature 6 weeks after infection had been initiated, and 48 hr. later these rats were killed by overdose of ether. Bacterial counts were performed on both kidneys and heart blood. The remaining 8 rats in the first group were used as a control. At 6 weeks they were subjected to a similar operative procedure except that the left ureter was merely mobilized but not tied. Thereafter they were treated in the same way as the rats with the occluded left ureter. The 14 rats in the second group were treated in the same way as the first group except that occlusion of the ureter (7 rats) or control operation (7 rats) was performed 3 months after infection was induced instead of 6 weeks.

RESULTS

The results are summarized in Fig. 1 and 2.

It can be seen that in the control animals of the 6 weeks group (Fig. 1*a*) right and left kidneys had organism counts of 10^5 or less except for 3 right kidneys which

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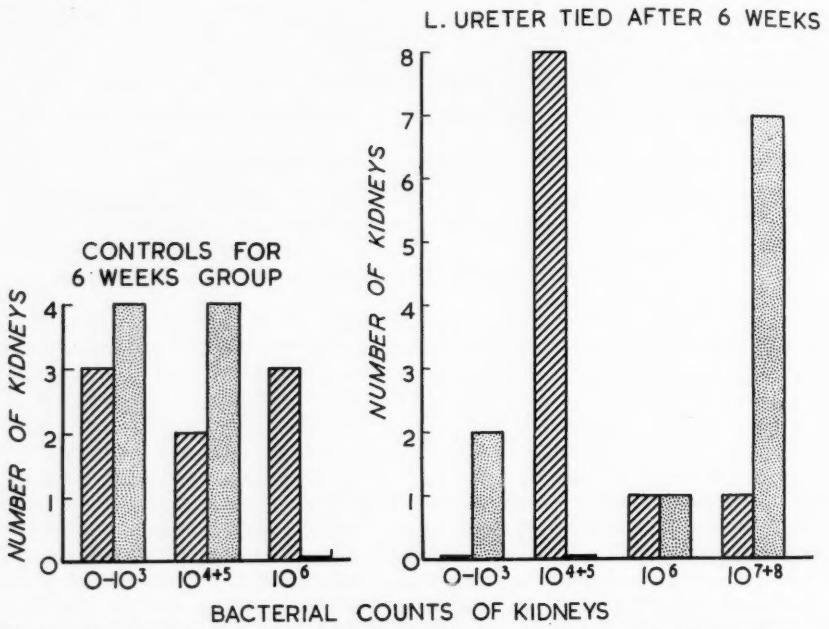

FIG. 1*a*.—Six week control group. Abscissa shows ranges of bacterial counts of kidneys, and ordinate shows numbers of kidneys within each range. *b*. Group with left ureter tied at 6 weeks.

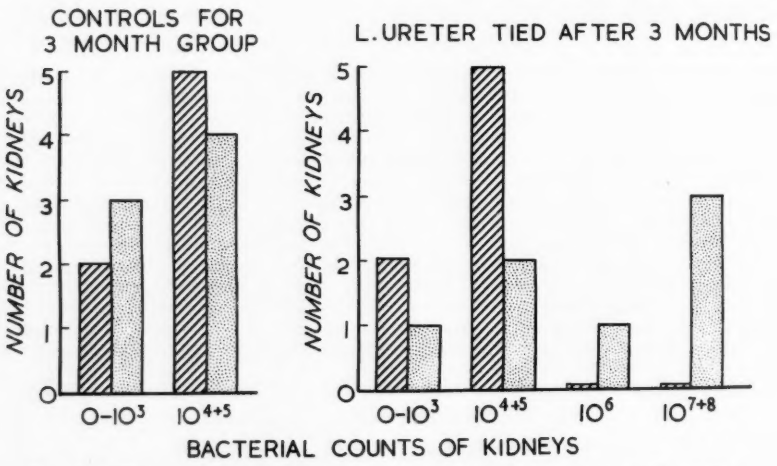

FIG. 2*a*.—Three month control group. *b*. Group with left ureter tied at 3 months.

had counts of the order of 10^6. Grossly these kidneys showed several discrete cortical scars except that in addition the 3 right kidneys with the high counts showed yellow streaking from the scars towards the papilla. In contrast the left kidneys whose ureters were occluded at 6 weeks (Fig. 1*b*) showed organism counts of over 10^7 in 7 out of 10 instances, but only 1 of the non-obstructed right kidneys had a count of this order. Grossly the 7 obstructed left kidneys showed small abscesses in relation to the cortical scars and the single right kidney with a very high count showed triangular shaped areas of infection in the cortex and outer medulla. The obstructed left kidneys with low counts showed no cortical scarring and the remaining right kidneys showed discrete cortical scars as in the right and left kidneys of the control group.

In the control animals of the 3 months group (Fig. 2*a*) both right and left kidneys showed uniformly low counts and cortical scars were usually present with no gross evidence of acute infection. In the rats where left ureteric occlusion was performed at 3 months (Fig. 2*b*), 3 out of 7 left kidneys had organism counts of over 10^7 and one additional left kidney had a count of between 10^6 and 10^7. The remaining obstructed left kidneys and all the non-obstructed right kidneys had counts of 10^5 or less. Grossly the left kidneys with high counts showed abscesses in relation to old cortical scars, while the remaining left kidneys and all the right kidneys showed cortical scars only. It should be noted that the left kidneys which failed to develop a high bacterial count after ureteric occlusion at 3 months showed cortical scars whereas those at 6 weeks which failed to develop high counts after similar treatment had no scarring.

Histological study of 2 non-obstructed right kidneys 3 months after infection, whose estimated bacterial count based on culture of half the kidney was 10^5 organisms, showed scarred areas in which normal glomeruli were crowded together because of loss or atrophy of proximal convoluted tubules. Some tubules in these areas persisted and a certain number contained polymorphs, while lymphocytes were seen in the interstitium. In 3 non-obstructed kidneys examined at 6 weeks with estimated counts of $1{\cdot}5 \times 10^5$ a similar picture was seen except that more tubules contained polymorphs, and polymorphs were present in the interstitium. Study of 2 left kidneys obstructed at 3 months showed similar scarred areas to those described, but in addition showed large numbers of interstitial polymorphs and polymorphs in tubules in areas adjacent to the scars.

Blood cultures were invariably sterile.

DISCUSSION

The results show that when infection is induced in the kidney by temporary occlusion of the ureter for 24 hr. coupled with intravenous injection of *Bact. coli*, healing takes place with the formation of discrete scars. When the left ureter is obstructed at either 6 weeks or 3 months after the initial induction of infection, there is a recrudescence of infection in a high proportion of kidneys in the former group and in a smaller yet significant proportion in the latter group. In the 6 weeks group those failing to develop severe infection showed very little or no scarring, but in the 3 months group some of those failing to show reactivation had definite scarred areas. The limited histological study showed that certainly at 6 weeks and to a lesser extent at 3 months in the control kidneys, there was evidence of active inflammation as judged by tubules containing polymorphs

amidst the more chronic changes. The presence of such active foci, although of minor degree, suggested the possibility of a reactivation of infection, if the appropriate stimulus were applied. Obstruction could supply such a stimulus in several ways. In the first place the free drainage of a small active focus might be prevented by the obstruction. Secondly the well established slowing of the renal circulation by obstruction of the ureter (Idbohrn and Muren, 1956), might permit the proliferation of organisms, and thirdly the defence mechanisms might be disturbed by changes in the local biochemical environment (Beeson and Rowley 1959). The failure of ureteric obstruction to cause reactivation in certain 3 month animals whose kidneys showed scarring could be explained by the presence of complete healing with no active focus. We have no histological evidence to substantiate this as the experiment was primarily concerned with the bacteriological aspect of the problem. The failure to cause acute infection in certain 6 weeks animals is in conformity with the absence of scarring in the obstructed kidney.

Whatever the mechanisms responsible for the reactivation of infection, the implications in human disease are apparent. The well known tendency for acute pyelonephritis to be recurrent is usually explained by a fresh invasion of organisms, and experimental studies indicate that small areas of scarring in the kidney, particularly in the medulla (de Navasquez, 1956 ; Rocha, Guze, Freedman and Beeson, 1958) render a kidney which has once had pyelonephritis more susceptible to re-infection. It is unfortunate that in the human we have very little knowledge of the pathological state of the kidney in the clinically quiescent phase between attacks of acute pyelonephritis. If it could be shown that there were small persistent foci of active infection, then it would not be unreasonable to postulate that a transitory interference with drainage of urine might produce a clinically overt attack of acute pyelonephritis.

SUMMARY

Acute infection was induced in both kidneys of the rat by temporary occlusion of the ureter combined with intravenous injection of *Bact. coli*. This infection was allowed to heal for periods of 6 weeks and 3 months, at which times the left ureter was completely occluded and the animals killed 48 hr. later. Organism counts of the kidneys showed that a reactivation of the infection took place in a high proportion of the rats at 6 weeks and in a smaller yet significant proportion of rats at 3 months.

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REGENERATION OF THE TISSUE MAST CELL: AN AUTORADIOGRAPHIC STUDY

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PRESENT knowledge of mast cell recovery following disruption by the histamine liberator compound 48/80 (a condensation product of *p*-methoxyphenethylmethylamine and formaldehyde) rests on the observations of Riley and West (1955) and Fawcett (1955) who believe that new mast cells are formed from small precursors in the adventitia of blood vessels. Conversely Dalgaard and Dalgaard (1948) think they originate from epithelial cells and migrate thence to their "characteristic" perivascular location. This study is an experimental examination of these concepts.

METHODS

Eleven adult male albino rats weighing about 250 g. were given intraperitoneal injections of compound 48/80 for 14 days. A twelfth animal from the same batch was kept intact. The starting dose was 100 μg. On the 2nd, 3rd, 4th and 5th days the doses were 200, 300, 500 and 750 μg. respectively until by the 6th day 1 mg. per day was being given. The injections were stopped on the 14th day and one rat at a time was injected intraperitoneally with 500 μc. of ^{35}S-labelled sodium sulphate at the following intervals thereafter: 0, 1, 2, 3, 4, 7, 9, 11, 14, 16, and 22 days. The rats are identified by the numeral of the day of injection, *e.g.* R0, R9, R16, etc. The normal rat was injected on the 11th day. Each animal was killed 24 hr. later and mesenteric spreads were prepared on plain and gelatin dressed glass slides, the latter being used for stripping film autoradiography. One coarse and two fine grain films exposed for 30 days were taken from each animal.

The spreads for histological examination were stained for 30 sec. with 0·1 per cent aqueous toluidine blue, while after development the fine grain preparations were stained with 0·01 per cent stain for 5 min. Thereafter the slides were washed under running tap water, taken through xylol and mounted in DPX.

^{35}S utilization was estimated in 2 ways: (*a*) Low power examination of coarse grain films and rough assessment of the cluster of activity over the whole spread on a scale 0 to 4+; (*b*) counting 50 mast cells in each fine grain preparation and noting how many showed ^{35}S uptake.

We did not consider it valid to estimate the activity of individual cells from a count of the emulsion granules associated with each cell.

RESULTS

After the 48/80 injections the animals showed the usual features attributed to the release of endogenous histamine, namely redness and swelling of the snout, ears and paws with varying degrees of prostration. These effects tend to lessen as the injections continue, but never entirely disappear.

The typical appearances of mast cell disruption were seen in the animals killed immediately and up to a few days after the injections of 48/80 had been

stopped (Riley and West, 1955). But even as early as one day after discontinuing injections there is evidence of incipient regeneration and by the 14th day this is probably complete. Regeneration begins simultaneously and diffusely throughout the preparation and is not particularly localized to perivascular regions (Fig.). Recovery is manifest in the increasing density of the granular aggregates and their adoption of cellular shapes.

There is no uniform pattern of sulphur utilization by the regenerating mast cell. Although there is a rough correlation between the results of the two methods of estimating ^{35}S uptake the Table shows how unreliable individual results can be.

TABLE.—*Degree of Radioactivity Due to Uptake of ^{35}S-labelled Sodium Sulphate in Mast Cells at Different Stages of Recovery after the Administration of Compound* 48/80

Rat	Estimated ^{35}S-uptake (coarse grain)	Number of M.C. showing radioactivity (50 counted)	
		Fine grain I	Fine grain II
R0	+++	9	47
R1	++++	26	44
R2	+++	50	46
R3	0	0	9
R4	++	10	43
R7	0	1	5
R9	++++	50	49
R11	+	8	9
R14	+++	41	44
R16	+	47	35
R22	0	36	9
Normal	++++	17	19

Thus rat R0 gave uptake counts of 9 and 47 cells out of 50 and even in any single slide considerable variation may be seen. Whether this is due to uneven contact between the tissue and the film or to some other cause, it excludes accurate quantitative comparison of preparations. The following qualitative observations can be made, however :

(i) Sulphur utilization in mast cells occurs diffusely throughout the mesenteric tissue and is not specially concentrated in perivascular areas. This corresponds with the histological evidence of the distribution of the regenerating cells.

(ii) Sulphur is taken up by " ghost cells ", that is by mast cells in a state of almost complete degranulation which can often only be detected and identified with difficulty and after some experience in the examination of such preparations. Not infrequently aggregates of radioactivity were seen although the underlying mast cell was scarcely discernible. As a general rule variation in radioactivity from cell to cell seemed to be unrelated to the degree of disintegration or recovery.

(iii) The utilization of sulphur is entirely or mainly a cytoplasmic function. Frequently the mast cell nucleus could be seen lying peripherally and in such a case the association of radioactivity with cytoplasm was obvious.

(iv) Accepting the limitations of the technique there still seems to be a real and considerable variation in the quantitive utilization of sulphur by the recovering mast cell. Whether this conforms to an intrinsic cyclical pattern or depends on general metabolic conditions involving the whole animal we cannot

say, but some allowance for spontaneous variation in cell activity has to be made in experiments like this. While it is possible that the mast cell has an increased avidity for sulphur during the first 2 or 3 days of recovery this could only be proved by a full scale experiment utilizing a large number of rats.

DISCUSSION

Although the tissue mast cell is profoundly disturbed by compound 48/80, even prolonged administration of this substance does not seem to destroy it, for 24 hr. after its administration has been stopped mast cell activity, as revealed by utilization of radioactive sulphur, can be observed throughout mesenteric tissue and not confined to perivascular areas. Although Riley and West (1955) distinguish between mast cell death and mast cell degeneration in their "acute" and "sub-acute" dosage experiments respectively, there is no convincing evidence that this is a real difference. All that one is entitled to conclude from their studies is that different dosage schedules of compound 48/80 produce different degrees of acuteness or severity of degranulation.

This kind of experiment therefore probably reveals nothing about the histogenesis of mast cells although providing information on their recovery and function. But even this may be unwarranted, for Riley, Shepherd and West (1955) have shown that in the rat, histamine liberators such as 48/80 act indeed, mainly to liberate histamine and have a less definite effect on the heparin content of mast cells and tissues. There is no certainty therefore that the utilization of sulphur, which is probably required for heparin and not histamine production, reflects only the needs of mast cell recovery and not to some degree also the ordinary turnover of heparin in the cells. It is this diversity of need which limits the validity of deductions about mast cell recovery based on sulphur uptake and which may account for the absence of a clear pattern in this study.

While mast cells do occur in chains alongside small vessels it is hardly correct to call this their characteristic location for they are seen throughout mesenteric tissue and also for example between myocardial fibres (Watson, 1958). The mature, fully granulated mast cell probably develops *in situ*. Given a viable cell, granule maturation can conceivably occur in any situation and there is no need to account for the presence of mast cells in areas remote from blood vessels by a theory of migration. It is possible that the time required for full maturation to occur is about 14 days and there seems to be virtually no or only a very short latent period of recovery following chemical trauma.

SUMMARY

An attempt to determine the pattern of mast cell recovery following degranulation by compound 48/80 using the uptake of ^{35}S-labelled sodium sulphate is described. The results are reported and the limits of their interpretation are discussed.

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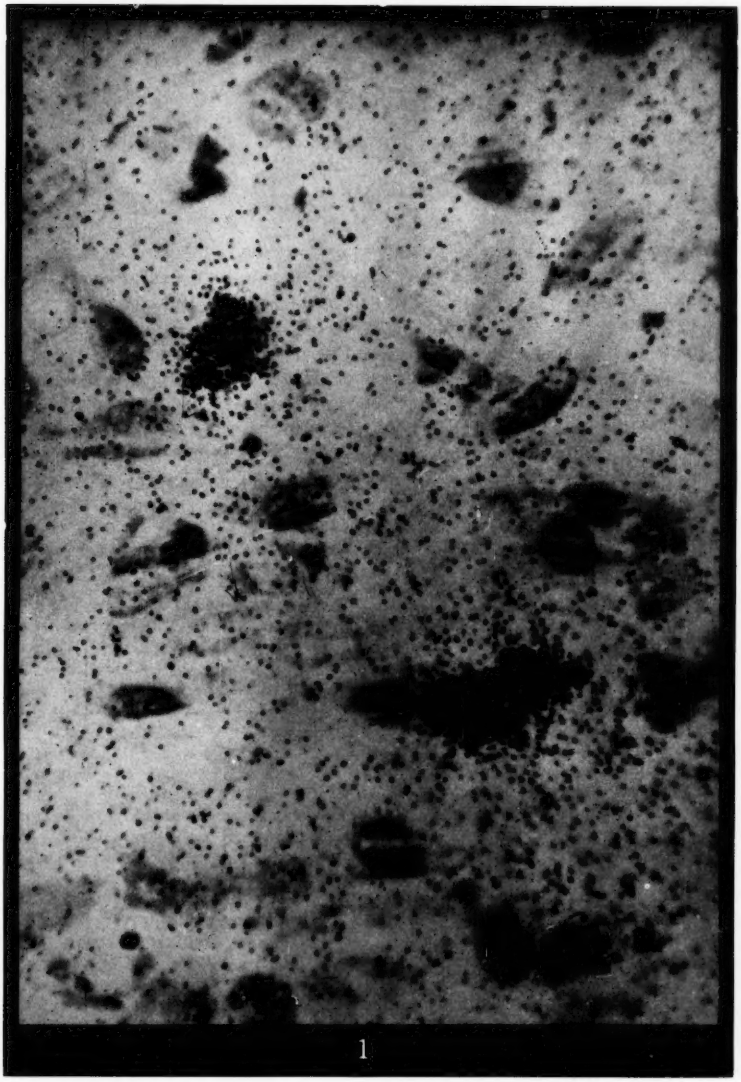
EXPLANATION OF PLATE.

FIG.—Photomicrograph of a fine grain autoradiograph of mesenteric spread from R2. The preparation has been stained with 0·01 per cent toluidine blue and shows ^{35}S uptake associated with recovering mast cells which are remote from mesenteric vessels. (×950.)



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THE PRODUCTION OF INTIMAL DEPOSITS OF LIPOID IN ISOLATED RABBIT'S AORTA

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THERE is still much controversy as to whether lipoid infiltration of the vessel is a primary or secondary phenomenon in atherogenesis. The experiments described here were made to study the primary aspect, by finding what effect cell-free lipoid suspensions would have on isolated preparations of normal artery. Since little or no active uptake or infiltration of lipoid occurred in slide-cell tissue-cultures, a method was devised of pulsating lipaemic sera and other fluids against the intima at a rhythm and pressure comparable to physiological arterial pulsation. A comparable technique with different apparatus was used by Evans, Brown and Ihrig (1957) to study the effect on the intima of the birefringent component of hypercholesterolaemic rabbit serum.

MATERIALS AND METHODS

Pulsation apparatus

Preliminary experiments were performed by tying 2 cm. tubular segments of rabbit's aorta to glass cannulae at each end. A cylinder of polythene was then slipped from the distal cannula over the segment of artery. Perfusion liquid was pulsated in the segment by a Dale-Schuster pump, against the resistance of a column of air at atmospheric pressure in the (sealed) distal cannula. This method, though promising, was wasteful as far as the arterial tissue and perfusing liquid were concerned, and the following method was devised for the purpose of testing small samples of tissue and liquid, with an adequate range of controls.

The pulsation chambers were made of Perspex (Fig. 1). The entire aorta from its valve to the renal arteries was excised, aseptically and without delay, from a rabbit killed by air-embolus. Segments 1 cm. long were cut, opened longitudinally between any tributaries, and freed of obvious adventitial fat; the excised segments were mounted severally in the chambers on sterile, precision-ground discs of stainless steel (A), with the intimal surface exposed to the pulsation compartment (B), which had a recess fitting exactly on to the steel plate. The adventitial surface was similarly exposed to a perfusion compartment (C) on the other side via a centrally-placed hole in the steel plate through which the arterial segment pulsated. The pulsation compartments, filled by the liquids under test, were connected to an air-pumping system (D) as described below. The adventitial compartments were separately connected to a circuit of tubes so that the same perfusing liquid passed from a common reservoir through one chamber to another. This enabled several perfusion-chambers to be flushed at once if leakages, chemical changes or infection developed, or if it was desired to vary the physical or chemical composition of the fluid in contact with the adventitial surface of the vessel. Oxygen was bubbled slowly through this circuit. The steel and Perspex surfaces in contact with the segments of vessels were treated with a non-fatty silicone vacuum grease and were leak-proof when lightly secured by 4 equalizing clamps (E).

Air under intermittent pressure was supplied to the pulsation chambers, in parallel, by a Dale-Schuster pump with 2 manifolds. To equalize the final pressure, these were led into a rose-distributor with 6 outlets, from which tubing conducted the air to each pulsation chamber at a pressure of 100–190 mm. Hg and a rhythm of 80–90 beats per min.

The pulsation chambers and the liquids in the apparatus were maintained at 37° throughout the experiments (F, G). Initially this was done by inserting the tissues into separate pulsation chambers in a thermostatically-controlled hot room. A heated box was subsequently devised to maintain a set of 6 pulsation chambers on an adjustable Perspex plate at the chosen temperature while the pump, motor and external connections were allowed to remain at room temperature. In this apparatus, 4 tests together with positive and negative controls could be included in each experiment. Between experiments the apparatus was sterilized in 1 per cent chlorhexidine in 10 per cent " Cetavlon " solution, followed by thorough washing in running water.

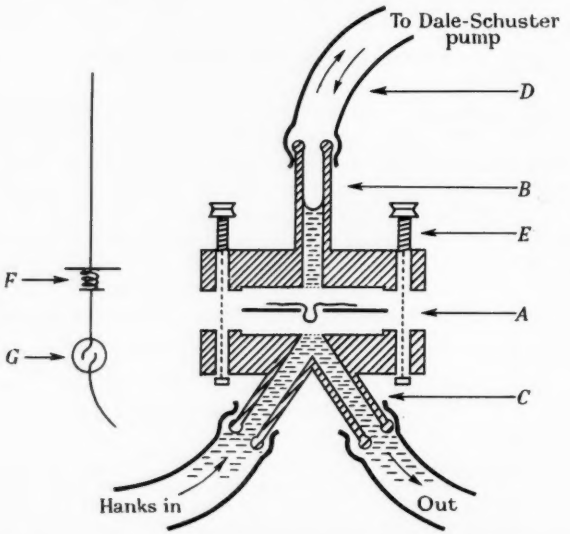


FIG. 1.—Exploded diagram of chamber used for pulsating lipoid-containing liquids against arterial segments. The complete apparatus contained 6 chambers on a Perspex plate mounted on a swivel joint.

A: Stainless steel plate, silicone-treated, carrying segment of vessel with intimal surface uppermost. B: Pulsation compartment containing liquid under test. C: Perfusion system supplying balanced salt solution and oxygen to adventitial surface of vessel. D: Rigid tubing supplying intermittent air pressure at 100–190 mm. Hg from Dale-Schuster pump. E: Clamping screws and nuts. F: Heating coil. G: Thermostat set at 37°.

Liquids used

(a) *For pulsation.*—On the intimal surface of the vessel, the pulsation compartments of the chambers accommodated 0·5–1·0 ml. of liquid. Normally 0·5 ml. was used, with a pulsating meniscus visible through the transparent Perspex. Various colloidal liquids were tested—normal and abnormal serums and plasmas, tissue extracts, artificial lipoid emulsions—as described in the results below.

As well as whole serum and plasma, fractionated sera were examined. Fractionation was performed ultracentrifugally in a Spinco rotor 40 with adaptors for 2 ml. lusteroid tubes. The low-density lipoprotein were separated by the preparative stage of the method of de Lalla and Gofman (1952) with minor adjustments in volume. Ultracentrifugation was carried out for 5 hr. at room temperature in a mean centrifuged field of 79,420 *g*. Lipoproteins with hydrated density $>1{\cdot}00$ $<1{\cdot}04$ g./ml. were removed by fine pipettes with the uppermost 0·5 ml. in the lusteroid tubes, and transferred to the pulsation-compartments of

the apparatus. As a control in these experiments, the high-density layer of the same serum was used.

(b) *For perfusion.*—The opposite, adventitial side of the vessel was continuously wetted by an oxygenated physiological solution. Usually, this was Hanks' balanced saline used routinely in our laboratories and having the following formula per litre : NaCl 8·00 g., KCl 0·40 g., $CaCl_2$ 0·08 g., $MgSO_4 7H_2O$ 0·20 g., $Na_2HPO_4 . 7H_2O$ 0·09 g., KH_2PO_4 0·06 g., Glucose 1·00 g., $NaHCO_3$ 0·35 g., Streptomycin 0·25 g., Neomycin 0·25 g., Fungizone 0·01 g., Penicillin G 250,000 units and Phenol red (0·1 per cent) 10·0 ml.

In any one experiment, one test liquid (*e.g.* hyperlipaemic plasma) could be tested against known positive and negative controls acting on segments of the same aorta ; conversely, segments of different aortas could be tested against the same liquid. The discs of aorta behaved as " semi-permeable" membranes, retaining the colloids in the pulsation compartment but permitting slight diffusion of crystalloids between the perfusion circuit and the pulsation compartments. Changes in the pH or transparency of the perfusate were immediately obvious through the transparent Perspex and connecting tubing.

Examination of tissue

After 24–96 hr. pulsation (usually 72 hr.) the segment of vessel was removed from the chamber and trimmed. The button-shaped central discs were inspected for visible deposits and then sectioned by various techniques—frozen unfixed and fixed, paraffin fixed, polystyrene (" Carbowax ") fixed. As a rule, fibrinous or lipoid deposits were best seen by fixing the disc briefly in Helly's solution before embedding and sectioning in polystyrene at room temperature, without further treatment. By this method, the losses and artefacts inherent in frozen and paraffin sections were avoided.

In effect, this technique provides a means of pulsating test liquid against the intima of a disc of aorta ; the pressure and periodicity of pulsation are regulated by adjustments to the pump and circuit ; the adventitial surface of the vessel is in contact with physiological solution to which oxygen and other substances can be added. This circuit is kept at atmospheric pressure, and the addition of antibiotics to the liquid normally suffices to prevent infection of the tissue during the usual period (72 hr.) of the experiments. The pulsation chambers and all liquids are maintained at 37° throughout the experiments.

RESULTS

Experiments with serum and plasma

Pulsation of normal human and rabbit serum and plasma produced little or no change in the exposed discs of aorta at the end of 96 hr. in the chamber (Fig. 2). The different layers—intima, media and adventitia—were well preserved and showed no lipoid or fibrinoid infiltration. The smooth muscle fibres of the media were loosened and more widely separated than in reference segments of aorta which had not been pulsated but seemed nevertheless to retain elasticity and tensile strength against pressures up to 200 mm. Hg.

Serum or plasma from hyperlipaemic, cholesterol-fed rabbits produced lipoid deposits in the intima and sub-intima when similarly pulsated, at the end of 72 hr. These deposits were superficial and localized (Fig. 3). They were strongly birefringent and partly sudanophilic. The birefringent material comprised minute splinter-shaped crystals, particles and spherulites, 0·5–2 μ in diameter, showing a cruciate interference pattern and optical activity in polarized light (Fig. 4). These particles were minute when viewed in sections freshly-cut from the carbowax-embedded discs, but some of them began to show some crystal-like growth *in situ* after a few days. The splinter-shaped and amorphous particles, but not the spherulites, were also seen readily in fixed and unfixed frozen sections. Treatment with organic solvents and paraffin processing, removed the birefringent and sudanophil material which was therefore lipoid in nature.

Experiments were then performed with ultracentrifugally fractionated sera from hyperlipaemic, cholesterol-fed rabbits. By this technique, it was found that the birefringent lipoid material which could be pulsated into the intima was present in the lipoproteins of hydrated density $>1{\cdot}00$ $<1{\cdot}04$ g./ml. No deposition occurred when the higher-density layers of serum were pulsated.

Experiments with other lipoids

In cholesterol-fed rabbits, the adrenal cortex and liver are rich in complex cholesterol-containing lipoid. Portions of tissue from these organs were homogenized in Griffiths' tubes in the inactive high-density fractions of ultracentrifuged normal rabbit serums in concentrations of approximately 10 per cent. After low-speed centrifugation to remove cellular debris, these homogenates were pulsated against aortic discs for 72 hr. Both types of homogenate produced lipoid intimal deposits, similar to those obtained with low-density lipoproteins from sera. Normal adrenal, similarly treated produced no change; normal, non-fatty liver produced no visible lipoid infiltration, though some fibrinous deposition was noted.

Among other lipoids so far tested, negative results have been obtained with normal human serum, serum of triglyceride lipaemia and with emulsions of human corpus luteum; triolein and cream triglycerides in 7 per cent albumin produced small sudanophilic globules in the superficial layers of the intima, without the complex components deposited from hypercholesterolaemic sera.

In all these experiments, the area of intima exposed to pulsation was that of a 5 mm. disc of aorta; the excursion on pulsation, at 120 mm. Hg, was about 2 mm. The lipoid infiltrations were therefore as a rule microscopic in size though, in the case of the lipoid-rich serum, or adrenal and hepatic homogenates from cholesterol-fed rabbits, visible deposits of about 1 mm. diameter were produced.

Comparison of pulsation-deposits and natural deposits of lipoid

The atheromatous deposits readily seen in the aortas of cholesterol-fed rabbits are characteristically superficial, sudanophilic and birefringent; under polarized light (Fig. 5) the birefringent particles are strikingly similar to those seen in sections of the aortic discs pulsated *in vitro* against hypercholesterolaemic serum as described above.

The question arises as to whether tissue pulsated *in vitro* for 72 hr. can be compared with a living vessel. This is partly answered by the absence of any comparable lipoid infiltration in control specimens pulsated for equal or longer

EXPLANATION OF PLATES

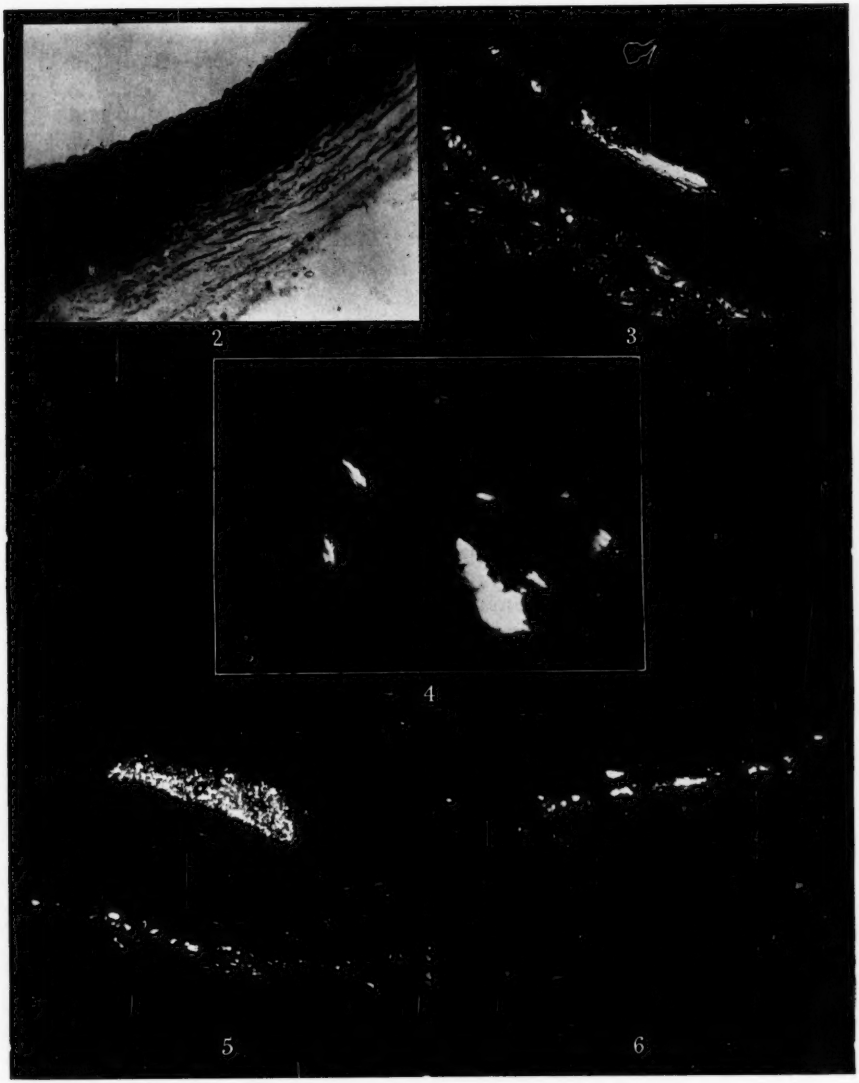
FIG. 2.—Segment of rabbit's aorta pulsated with normal rabbit's serum. ×67. Polarized light at half-extinction.

FIG. 3.—Segment of rabbit's aorta pulsated with hypercholesterolaemic rabbit's serum. ×67. Polarized light at half-extinction.

FIG. 4.—Segment of rabbit's aorta pulsated with hypercholesterolaemic rabbit's serum. Spherulites in intimal deposit. ×536.

FIG. 5.—Early atheromatous intimal deposit in aorta of a cholesterol-fed rabbit. ×67. Polarized light.

FIG. 6.—Early atheromatous deposit in intima of aorta in a child aged 2 yr. ×67. Polarized light.



Stewart.

periods against non-lipaemic serum, and by the fact that elasticity and structural boundaries were preserved during the period of experimentation.

A further question, which always arises when cholesterol-fed rabbits are the subjects of experiment, is to what extent superficial lipoid deposits can be compared to human atheromatous lesions. This question is clearly too complex to discuss here but it is relevant to the present results to show (Fig. 6) that the earliest forms of human atheroma, such as may sometimes be found in autopsies of children and young adults, are superficial and contain soft birefringent lipoid similar to that observed in rabbit vessels in the present study.

DISCUSSION

The objects of the present study were twofold; to see if cell-free lipoids in serum or other media were able to infiltrate isolated arterial tissue; and to devise a technique for studying the response of isolated arteries to various factors concerned in atherogenesis and its cure. The second objective clearly requires further exploration but the results reported here suggest that the first objective might have been attained. Under the conditions described, the abnormal forms of lipoid found in the serum, liver and adrenal of cholesterol-fed rabbits seem to be able to infiltrate and to some extent to penetrate the intima of isolated segments of normal rabbit aorta. This infiltration occurs in the absence of fibrinogen, red cells or leucocytes in the pulsating fluid, and shows some similarity in appearance to the lipoid deposits in the atheroma of rabbits with experimental cholesterosis and to the soft superficial lesions of early human atheroma.

A similar finding was reported by Evans, Brown and Ihrig (1957) who found that hypercholesterolaemic rabbit serum produced birefringent deposits when pulsated against excised aorta. The substance responsible for deposition in their experiments, as in the present study, was associated with low-density β-lipoproteins which were absent in normal rabbit serum.

The results here obtained with homogenates of abnormal lipoid from liver and adrenal cortex as well as with serum show that, in cholesterol-fed rabbits, the substance deposited as atheroma in vessels is probably identical with that deposited in certain other tissues. There are reasons for believing (Stewart, 1959) that certain normal as well as abnormal lipoids are carried in plasma and tissue in a mesomorphic physical state, between true solid and liquid. This state is characterized, in the case of certain cholesteric complexes, by a liquid crystalline form of which the spherulites described above in the intimal deposits are examples. These spherulites, and other characteristic lipoid particles, are abundant in the soft intimal lesions of naturally-occurring and experimental atheroma. The presence of similar particles in the deposits produced *in vitro* in the present experiments suggests that such deposits may have some validity in the study of atherogenesis.

SUMMARY

A technique is described for pulsating cell-free lipoid fractions of serum, and other lipoids, against isolated segments of normal rabbits' aortas.

Pulsation of the low-density lipoprotein component of the serum of cholesterol-fed rabbits produces complex lipoid deposits in the intima of the isolated vessel.

Similar complex deposits are produced by pulsating abnormal lipoid complexes from certain tissues in such rabbits, but not by simple triglycerides in serum or in artificial emulsions.

The deposits described have features in common with the early stage of naturally-occurring and experimental atheroma.

The results suggest that early atheromatous deposition in the intima can be caused primarily by abnormal low-density lipoproteins in the serum.

Grateful acknowledgement is made to the following: Miss P. M. Harrison, for invaluable technical assistance; Dr. J. M. Barnes and Dr. P. D. Cooper, for facilities in their departments; Mr. R. C. Emery, for constructing the pulsation apparatus and Mr. R. F. Legg for the photomicrographs.

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THE INDEPENDENT PRODUCTION OF ATHEROSCLEROSIS AND THROMBOSIS IN THE RAT

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IN recent years dietary fat has been implicated as a factor in both atherosclerosis and ischaemic heart disease. But the hypothesis is founded more on assumption than fact (Bronte-Stewart, 1958) and conclusive experimental proof is so far lacking.

Animal experiments play an important part in attempting to elucidate the nature of the diseases since it is not possible to determine satisfactorily the presence of atherosclerosis in living man. Furthermore, it is not justifiable to assume that the presence of ischaemic heart disease reflects the extent or degree of atherosclerosis (Morris, 1951 ; Robertson, 1959).

Thomas and Hartroft (1959) fed to rats a diet containing butter, cholesterol, cholic acid and thiouracil and produced myocardial infarction and vasothrombosis. They emphasized that such lesions might occur without any demonstrable vascular disease. The investigations reported here are concerned with the type of vascular lesions produced by diets containing variable amounts of either arachis oil or butter. The two fats differ in that arachis oil has a high content of linoleic acid and that butter contains saturated acids of low carbon number (Table I).

TABLE I.—*Proportions (per cent molar) of Fatty Acids in Butter and Arachis Oil**

	Number of C atoms	Number of C—C double bonds	Butter	Arachis oil
Butyric	4	0	10	—
Hexa-, Octa-, Decanoic and Lauric	6, 8 10, 12	0	15	—
Myristic	14	0	10	—
Palmitic	16	0	20	15
Stearic	18	0	10	5
Oleic	18	1	30	40–60
Linoleic	18	2	5	20–35

* Hilditch (1956).

EXPERIMENTAL

Animals.—Laboratory bred piebald rats were fed a stock diet after weaning until about 100 g. body weight and then placed into experimental groups. Each animal was housed in a separate cage, fed food and water *ad lib.* and weighed weekly.

TABLE II.—*Design of Experiment and Diets Used*

Group	Diet factor investigated	Number of animals group	Sex	Variable constituents in diet† (g.) Cholesterol	Thiouracil	Cholic acid	Butter	Arachis oil	Sucrose
1	Diet known to produce myocardial infarction	25	M.	50	3	20	400	—	169
2	Substitution of arachis oil for butter	20	,,	50	3	20	—	400	169
3	Reduction of butter to 5 per cent	10	,,	50	3	20	50	—	469
4	Substitution of females for males	15	F.	50	3	20	400	—	169
5	40 per cent butter alone*	10	M.	—	—	—	400	—	242
6	40 per cent arachis oil alone*	10	,,	—	—	—	—	400	242
7	5 per cent butter alone*	20	,,	—	—	—	50	—	592
8	Omission of thiouracil	10	,,	50	—	20	400	—	172
9	Substitution of 10 per cent arachis oil for 40 per cent butter	10	,,	50	3	20	—	100	469
10	10 per cent arachis oil only*	10	,,	—	—	—	—	100	532

* i.e. cholesterol, cholic acid and thiouracil omitted.

† All diets included Casein 200, choline chloride 10, salts 40, cellulose powder 100, magnesium oxide 5, inositol 2 and B vitamins (thiamine 16 mg., riboflavin 16 mg.,pyroidoxine HCl, 16 mg., folic acid 10 mg., calcium pantothenate 40 mg., biotin 0·6 mg., B_{12} 0·05 mg., nicotinamide 0·2 g.).

TABLE III.—*Average Body Weight of Rats After* 5, 10 *and* 20 *Weeks on Experimental Regime, and Average Survival Times*

Group	Factor investigated	Number of animals/ group	Sex	Average body weight (g.) at (weeks) 0	5	10	20	Number surviving at 20 weeks	Average survival (days)
1	Diet known to produce myocardial infarction	25	M.	120	102	112§	—	0	95
2	Substitution of arachis oil for butter	20	,,	102	102	97§	—	0	113
3	Reduction of butter to 5 per cent	10	,,	102	122	—	—	0	44
4	Substitution of females for male	15	F	108	109	98§	—	0	72
5	40 per cent butter alone	10	M.	101	274	390‡	471*	10	*
6	40 per cent arachis oil alone	10	,,	120	291	382‡	430*	10	*
7	5 per cent butter alone	20	,,	102	250	316	366	10	*
8	Omission of thiouracil	10	,,	102	221	275‡	313*	8	†
9	Substitution of 10 per cent arachis oil for 40 per cent butter	10	,,	115	122	121§	—	0	100
10	10 per cent arachis oil cnly	10	,,	103	253	334	401	10	*

* All survived.

† One died on 64th the other at the 56th day.

Statistics: Groups compared with group 10 and 20 weeks.

‡ $0{\cdot}05 > P > 0{\cdot}01$. § $P < 0{\cdot}001$.

Experimental design.—The diets fed are shown in Table II and were variations of that used by Thomas and Hartroft (1959) which was claimed to produce a high incidence of myocardial infarction. They were designed to investigate the following points: (i) the replacement of butter by arachis oil; (ii) the reduction of concentration, of butter to 5 per cent; (iii) replacement of males by females; (iv) the omission of cholesterol, cholic acid and thiouracil; (v) the omission of thiouracil alone. Animals receiving 5 per cent butter and 10 per cent arachis oil were used as controls. When a constituent of the diet was omitted, an equivalent weight of sucrose was added. The diets were prepared once weekly and in those containing a high concentration of fat, every attempt was made to avoid rancidity. Each rat was given one weekly supplement comprising 1000 i.u. vitamin A and 40 i.u. vitamin D, supplied as one drop of halibut liver oil, 2 mg. α-tocopheryl acetate in 2 drops arachis oil and 0·05 mg. 2-methyl-1 : 4 naphthoquinone in one drop arachis oil.

Electrocardiograms.—These were taken according to the method of Drury, Harris and Maudsley (1930) using an instrument of the mirror type manufactured by the Clifton Instrument Co., Cambridge. A standard lead (S2) and two unipolar leads (corresponding to the V1 and V2 positions in man) were taken of all animals in groups 1–4 fortnightly and of 5 animals in each of the groups 5–9 at least once a month.

Pathology.—Necropsy was made as soon as possible after death, the time interval varying from ½ hour to 3–4 days. If there was a time lag before death and necropsy, carcasses were stored in the refrigerator. Animals surviving 20 weeks were killed by ether anaesthesia. Specimens were taken of the heart, thoracic and abdominal aorta, kidneys, adrenals, liver, lungs, testes, skin, thyroid and spleen. Fixation was in all cases by neutral buffered formol saline. Frozen sections were stained by Oil red O and paraffin sections by a variety of conventional histological and histochemical methods in order to demonstrate special features.

Oil injection method.—Selected animals were killed by ether anaesthesia and the coronary vascular tree injected with radio opaque oil (neohydriol, iodized poppy-seed oil) by means of a cannula in the thoracic aorta; all other vessels coming off the aorta above the cannula were ligated. Using this method it was possible to study the macroscopic appearance of the coronary tree by X-ray photography and the microscopic appearance by frozen sections stained with oil red O; neohydriol is strongly sudanophil.

RESULTS

Body weights

The average weight of the different groups at 5, 10 and 20 weeks are given in Table III. Inclusion of thiouracil in the diet suppressed growth (groups 1–4 and 9) and the animals just maintained their body weight throughout the experiment. Rats consuming 40 per cent butter or 40 per cent arachis oil alone (groups 5 and 6) were obese and had higher body weights than the controls receiving 5 per cent butter and 10 per cent arachis oil alone (groups 7 and 10).

Mortality

As shown in Table II, animals in groups 1 and 2 survived for about the same time (14–15 weeks). Group 3 survived for only 6 weeks. Compared with animals in group 1, females (group 4) succumbed before males. Only those animals receiving thiouracil had a high mortality as in other groups most were living after 20 weeks.

Electrocardiograms

Compared with man the ST segment of the rat cardiogram is considerably elevated. Although a standard lead S2 and unipolar leads V1 and V2 were taken, it was found that unipolar lead V2 gave the most useful information. Among the experimental groups, abnormal patterns were seen in 5 animals out of 25 in group 1 (males) and 1 animal out of 15 in group 4 (females). Abnormalities consisted

of inverted T waves. As to be described later all animals with myocardial infarction had abnormal electrocardiograms. Such patterns were seen only in groups 1 and 4.

Pathology

In groups 1 and 4, the apex of the heart invariably showed a small patch of fatty material and occasionally a larger area of necrosis (Fig. 1). The lungs were congested and showed generalized oedema. In group 2, the patch at the apex of the heart was either minute or absent and the lungs were not congested.

Rats receiving cholesterol and cholic acid (groups 1–4, 8 and 9) had intensely fatty livers and the plasma contained a very high content of lipid so that vessels were often conspicuously white after death. As expected all animals receiving thiouracil had generalized symmetrical enlargement of the thyroid.

Microscopic examination

Occurrence of thrombosis.—In 8 animals in group 1 and 2 in group 4, large thrombi were seen in either the ventricles, atria, the thoracic aorta, or renal veins. Fig. 2 shows a thrombus in the left ventricle attached to an apical patch of lipid filled macrophages. Fig. 3 shows thrombi occurring simultaneously in the left atrium and left ventricle. Fig. 4 shows an endothelialized thrombus in the aorta and Fig. 5 in the renal vein. Thrombi were also attached to areas of infarction when these occurred (Fig. 6). No thrombi were seen in any group other than groups 1 and 4 (Table IV).

Occurrence of infarction.—Six animals in group 1 and one in Group 4 showed recent or partially healed myocardial infarction in either the left ventricular wall or ventricular septum. It appeared likely that luminal thrombi were the cause

TABLE IV.—*Distribution of Lesions*

Group	Description	Number of animals/ group	Number of animals showing lesions				
			Athero-sclerosis	Abundant endothelial lipid filled macrophages	Throm-bosis	Infarction	
						Heart	Kidney
1	Diet known to produce myocardial infarction	25	0	25	13	6	2
2	Substitution of arachis oil for butter	20	12	0	0	0	0
3	Reduction of butter to 5 per cent	10	0	0	0	0	0
4	Substitution of females for males	15	0	8	4	1	2
5	40 per cent butter alone	10	0	0	0	0	0
6	40 per cent arachis oil alone	10	0	0	0	0	0
7	5 per cent butter alone	10	0	0	0	0	0
8	Omission of thiouracil	10	0	2	0	0	0
9	Substitution of 10 per cent arachis oil for 40 per cent butter	10	3	0	0	0	0
10	10 per cent arachis oil only	10	0	0	0	0	0

of the infarction since organization in the thrombi was far advanced beyond that in the infarcts. Such thrombi produced infarction by either blocking ventricular-arterio-luminal vessels or, if present in the aorta by blocking coronary ostia. One rat which had an endothelialized thrombus in the aorta (Fig. 4) showed an abnormal electrocardiogram with an inverted T wave for some 4 weeks ; this became normal about a fortnight before death. It is possible that the aortic thrombus caused intermittent obstruction to coronary blood flow. All rats showing cardiac infarcts had abnormal electrocardiographic patterns.

A few animals were investigated using the oil injection method described above. No significant differences from normal were seen in group 1.

Two animals in group 1 and two in group 4 showed renal infarction. Both animals had thrombi in the renal veins ; it appeared likely that infarction was due to venous and not arterial thrombosis.

No other groups showed either myocardial or renal infarction (see Table IV).

Lipid changes in the vascular system

Groups 1 and 4 showed some accumulation of lipid filled macrophages particularly on the ventricular endocardial surface of the base of the mitral cusp (Fig. 7) and at the apex of the heart (Fig. 6). Small areas of intimal sudanophilia were seen in the abdominal aorta of 3 animals in group 1. This hardly amounted to atherosclerosis. Fig. 8 shows a typical section of aorta from this group and the absence of lipid infiltration.

In contrast, animals in group 2 showed atherosclerotic plaques in the thoracic aorta (Fig. 11) and coronary arteries (Fig. 9 and 10) which closely resembled those of human atherosclerosis (Fig. 12). We define atherosclerosis as a raised lesion capped by a fibro-elastic tissue beneath which is amorphous debris containing cholesterol crystals and other sudanophil lipid ; the underlying elastica being often fragmented. Lesions were seen mainly in the proximal aorta but none was seen in the abdominal aorta or renal arteries. Group 9, in which arachis oil was reduced from 40 per cent to 10 per cent was the only other group to show atherosclerotic lesions. The lesions were much smaller in size however and occurred in a smaller proportion of animals. No other groups showed similar changes (Table IV).

Lipid changes in the organs

Rats fed cholesterol and cholic acid (groups 1–4, 8 and 9) had large numbers of lipid filled macrophages in renal glomeruli, zona glomerulosa and reticularis of the adrenal, the red pulp of the spleen, in the pulmonary alveoli and in pleural tags of the lungs. Lipid in the lungs was found in all animals fed fat and probably arrived there by inhalation ; this is supported by the frequent presence of foreign body giant cells in relation to such lipid. The adrenal, of group 4 (females) showed more lipid than group 1 (males). The same groups of animals had intensely fatty livers and showed the presence of large numbers of cholesterol crystals when examined under ordinary light and polarized light. The deposition of cholesterol crystals was more extensive in group 2 than in the other groups. In addition, group 1 showed widespread hepatic cell vacuolation some of which was due to lipid material.

Other changes observed

Lungs from animals in groups 1 and 4 showed intense oedema, emphysema and congestion thus confirming the macroscopic findings. Other groups had moderate or no visible oedema.

Calcinosis of the arterioles of the thyroid and kidney were seen in all groups fed cholesterol and cholic acid. Two animals had large stones in the renal pelvis.

Thyroid glands.—Animals receiving thiouracil showed conspicuous hyperplasia. A curious finding was the occasional presence of colloid droplets in the acini.

DISCUSSION

In man, coronary thrombosis and atherosclerosis are usually associated clinically. Moreover it is recognized that the diseases are more prevalent in the obese (Mann and Stare, 1954). Our results indicate that thrombosis, atherosclerosis and obesity can be apparently produced independently using 3 different experimental regimes. A diet containing 5 per cent cholesterol, 2 per cent cholic acid, 0·3 per cent thiouracil and 40 per cent butter (Thomas and Hartroft, 1959) produced thrombosis, myocardial and renal infarction but no significant atherosclerosis. In contrast, replacement of the 40 per cent butter with 40 per cent arachis oil produced vascular lesions which closely resembled those of human atherosclerosis but no thrombosis or significant infarction. Animals fed 40 per cent butter alone or 40 per cent arachis oil alone became obese but developed none of these lesions.

But coronary thrombosis and atherosclerosis are more prevalent in countries with a high intake of animal fat (Bronte-Stewart, 1958). Sinclair (1956) has related the incidence to a relative deficiency of essential fatty acids which are present in only small amounts in most animal fats. Our results are difficult to reconcile with this hypothesis. Whilst it is true that the thrombosis was produced by the diet containing butter, atherosclerosis occurred only in those animals consuming arachis oil which contains a relatively high amount of essential fatty

EXPLANATION OF PLATES

FIG. 1.—Extensive infiltration of lipid at cardiac apex. Group 1.

FIG. 2.—Thrombus adherent to left ventricular apex. Group 1. H. & E. ×19.

FIG. 3.—Atrio-ventricular junction of heart showing mitral valve cusp diagonally placed with thrombus in left atrium (A) and in left ventricle (B). The ventricular septum is on the bottom right (C). Group 1. H. & E. ×19.

FIG. 4.—Endothelialized thrombus lying free in aorta. Group 1. H. & E. ×19.

FIG. 5.—Transverse section of renal pelvis showing thrombus in renal vein. Group 1. H. & E. ×29.

FIG. 6.—Thrombus adherent to left ventricular apex. Thrombus (T) in left ventricle; normal muscle (N); infarct (A); area of lipid infiltration (B). Group 1. H. & E. ×15.

FIG. 7.—Section through base of mitral cusp showing lipid filled macrophages on the ventricular endo-cardial surface. Group 1. H. & E. ×56.

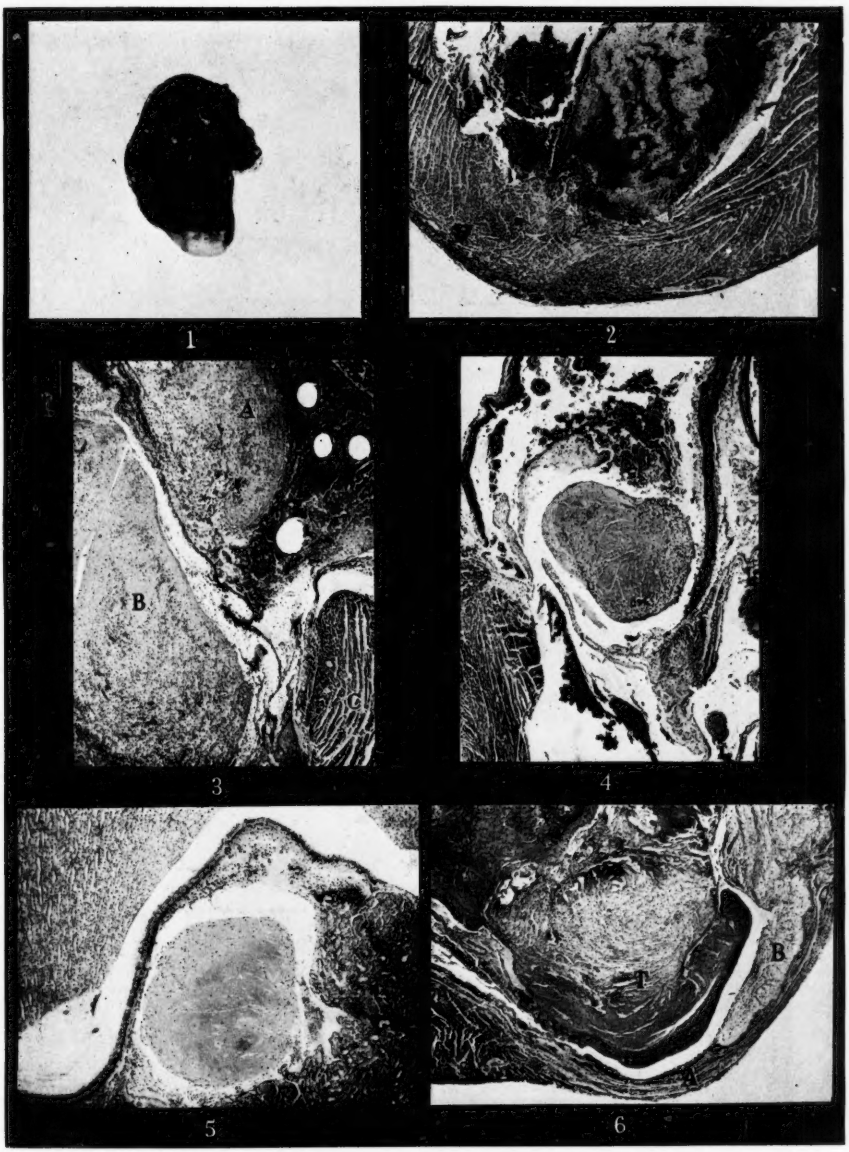
FIG. 8.—Aorta. Note one small area (W) of inner medial lipid infiltration. (M) Group 1. Oil red O. ×22.

FIG. 9.—Aorta (A) and coronary arteries (B and C) one of which (C) shows an atherosclerotic plaque. Group 2. H. & E. ×17.

FIG. 10.—Coronary artery as shown in Fig. 9. Group 2. H. & E. ×52.

FIG. 11.—Aorta. (A) showing an atherosclerotic plaque. Group 2. H. & E. ×17.

FIG. 12.—Atherosclerotic plaque showing sudanophil lipid and a cap of fibro-elastic tissue. Oil red O. ×56.



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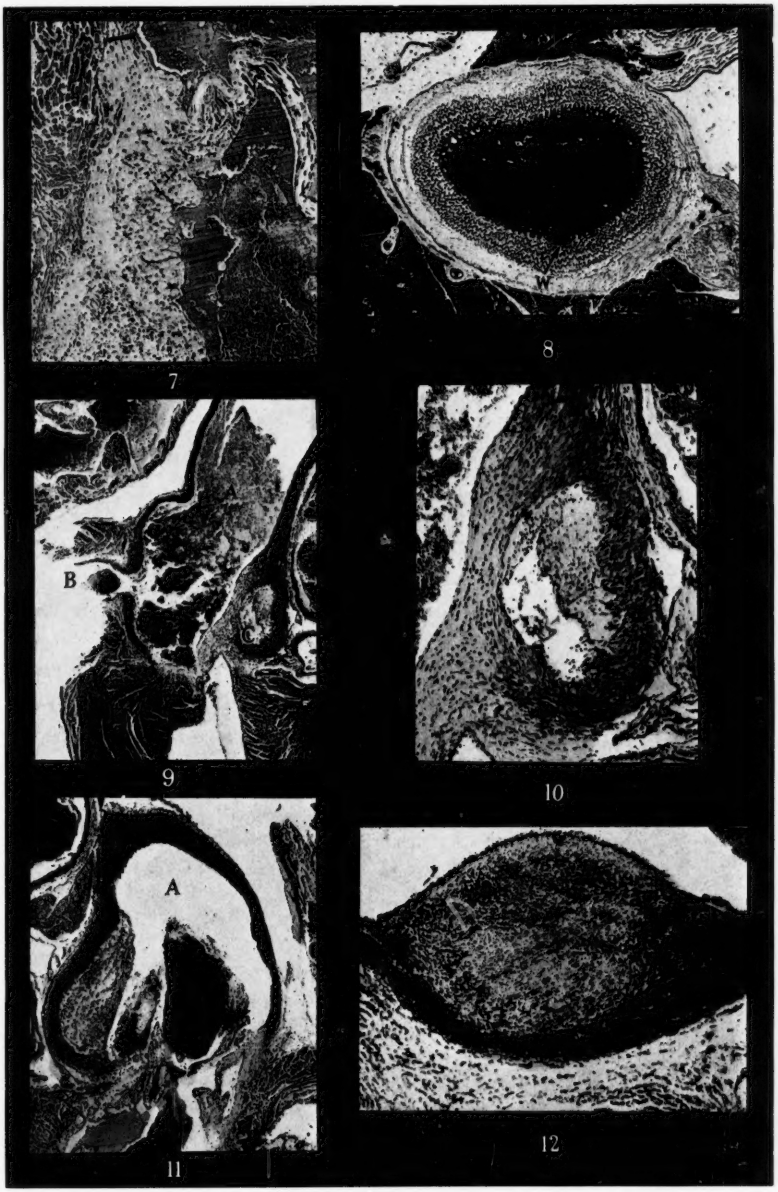

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acid, linoleic acid (25 per cent). Further work is obviously necessary to ascertain which constituents of either fat are responsible for the lesions observed.

Death in male rats fed the diet containing 40 per cent butter, cholesterol, cholic acid and thiouracil (group 1) was apparently the result of extensive thrombosis in the aorta, ventricles and atria leading to cardiac failure and subsequent congestion of the lungs. Myocardial infarction was probably related to the thrombosis and caused by obstruction to the arterio-luminal sinusoids. Failure to find arterial lesions constantly related to thrombi suggested that thrombosis was a result of changes in the properties of the blood either by an increase in coagulability or by decreased fibrinolysis. Moreover thrombosis was not confined to arteries but also involved the renal vein, and the obstruction caused thereby was no doubt responsible for the renal infarction observed. Electrocardiograms in rats with myocardial infarction were clearly abnormal and the technique recommends itself for use in future experiments since progress can be observed without killing the animal. More detailed studies of the electrocardiograms are to be presented elsewhere (Hill, Howard and Gresham, 1960).

All the above mentioned constituents in the diet appear to be essential for thrombosis. Thus the omission of thiouracil (group 8) or the reduction of the concentration of butter to 5 per cent (group 3) prevented thrombosis. Animals in the group with thiouracil omitted survived the experiment but the group in which the butter was reduced to 5 per cent lived only 6 weeks. Females (group 4) appeared to be less susceptible to thrombosis and to the infarction than males (group 2) but it should be noted that their survival time was somewhat less, and hence they were on the dietary regime for a shorter period.

The cause of death was not determined in the animals fed 40 per cent arachis oil, cholesterol, cholic acid and thiouracil. Cardiac failure seemed unlikely as the prime cause since the lungs were not usually congested and oedematous. A high intake of arachis oil apparently favoured the production of the atherosclerosis observed since fewer and less conspicuous lesions were seen when its concentration was reduced to 10 per cent (group 9). The atherosclerotic lesions closely resembled those seen in man and were not associated with thrombosis. Serum cholesterol values in the 2 groups showing atherosclerosis were not higher than the other groups (Howard and Gresham, unpublished). A characteristic finding, however, was a greater deposition of cholesterol in the liver than in other groups and the liver cells were packed with cholesterol crystals.

As might be expected in those groups consuming cholesterol and cholic acid, large quantities of lipid were taken up by phagocytic cells particularly in suprarenals, spleen, and lungs. Livers of these animals were intensely fatty and the lipid consisted chiefly of cholesterol esters. In many of these animals, arterial calcinosis occurred particularly in the kidney, thyroid and lungs. The mechanism of calcinosis appears obscure and would seem to warrant further study.

All the rats fed on diets containing 40 per cent butter alone, 40 per cent arachis oil alone, 5 per cent butter alone or 10 per cent arachis oil alone survived the experiment and some of these rats are being allowed to continue. It is hoped to give a more detailed report later. Animals examined after 4–6 months had developed no atherosclerotic or thrombotic lesions. It is difficult to extrapolate experimental data obtained in the rat to the occurrence of the diseases in man. Our data do not necessarily provide evidence that a high fat diet *per se* is the primary cause of atherosclerosis or ischaemic heart disease. On the other hand

it suggests that the type of fat consumed may have a major influence on the type of lesion produced.

SUMMARY

Rats were fed a diet containing 5 per cent cholesterol, 2 per cent cholic acid, 0·3 per cent thiouracil and variable amounts of butter or arachis oil. A pathological study was made of the heart, aorta and selected organs.

Only rats fed the above-mentioned diet containing 40 per cent butter had a high incidence of myocardial and renal infarcts and abnormal electrocardiograms. The infarction was attributed to thrombotic occlusion of arterioluminal sinusoids in the ventricles and of the renal veins in those with renal infarcts. Atherosclerosis was not seen.

Substitution of 40 per cent arachis oil for 40 per cent butter produced neither infarction nor thrombosis. In contrast, extensive atherosclerosis occurred in the thoracic aorta and coronary arteries and was similar to that seen in man. Atherosclerosis was less severe if the arachis oil content of the diet was reduced to 10 per cent.

Rats fed 40 per cent butter or 40 per cent arachis oil alone became obese but developed no vascular, renal or myocardial lesions.

All diets containing cholesterol and cholic acid produced arterial calcinosis and large numbers of lipid filled macrophages in most organs.

It was concluded that thrombosis, atherosclerosis and obesity are three independent phenomena.

We wish to thank M. A. Ward, Mr. S. G. Impey and Mr. A. J. King for their valuable technical assistance, and Mr. S. W. Patman for preparing the photomicrographs.

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THE EFFECT OF MACROCYCLON ON THE MULTIPLICATION OF TUBERCLE BACILLI IN THE LUNGS AND SPLEEN OF MICE*

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CORNFORTH, HART, REES AND STOCK (1951) first reported that a commercial non-ionic surface active agent "Triton WR 1339", and other synthetic macromolecular products, suppressed the development of experimental tuberculosis in the mouse. This action of "Triton WR 1339" on experimental murine tuberculosis was confirmed by Solotorovsky and Gregory (1952) who also noted that this compound acted synergistically with dihydrostreptomycin. Subsequently, Rees (1953) noted that these surface active agents can produce regression and healing of tuberculous infection in guinea-pigs; findings which have been confirmed by Kato and Gozsy (1957). Rees (1957), furthermore, has demonstrated a favourable effect on the course of *Mycobacterium lepraemurium* infection in mice.

The mechanism whereby these surface active agents bring about a marked suppression of experimental tuberculous infections has not been elucidated. It has been suggested by Lovelock and Rees (1955) that these compounds might remove preferentially the more hydrophobic lipids of the tubercle bacilli. This action conceivably could render the tubercle bacilli more sensitive to destruction within the mononuclear phagocytic cells. This theory of the mode of action of "Triton WR 1339" and related compounds is based upon the following direct observations. These surface active agents exhibit *in vitro* no inhibitory effect on the multiplication of tubercle bacilli nor are they converted to bacteriostatic or germicidal substances *in vivo* (Cornforth, Hart, Nicholls, Rees and Stock, 1955; Parlett and Youmans, unpublished); they have been shown to accumulate in relatively high concentration in macrophages, the cells within which tubercle bacilli most commonly reside and multiply (Lovelock and Rees, 1955; Rees, 1958); it has been observed that macrophages obtained from animals treated with "Triton WR 1339" have a greater capacity to inhibit the multiplication of tubercle bacilli in tissue culture than do macrophages obtained from untreated animals (Mackaness, 1954). Furthermore, the surface of the tubercle bacilli apparently is affected since in the presence of "Triton WR 1339" and macrocyclon the oxidation of substrates by tubercle bacilli *in vitro* can be appreciably increased (Hart and Rees, 1955; Youmans and Youmans, unpublished).

Although the foregoing evidence strongly suggests that these non-ionic surface active agents affect favourably the course of tuberculous infection in animals by altering the tubercle bacillus so that it is more amenable to the destructive mechanisms of the macrophage, no data is available relative to the

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actual fate of tubercle bacilli in the organs of animals treated with these surface active agents. The experiments to be presented here were undertaken to obtain a definitive picture of when, and to what degree, the multiplication and viability of tubercle bacilli *in vivo* is affected.

MATERIALS AND METHODS

Macrocyclon, the surface active agent used in these studies, is a macrocyclic compound, a tetracyclic condensation product derived from *p-tert*-octyl phenol and polymerized with ethylene oxide. Each phenolic nucleus has an average of 12½ ethylene oxide units. It differs from " Triton WR 1339 " in being a cyclic instead of a linear condensation product and furthermore the tetracyclic octyl phenol intermediate is obtained in a pure and crystalline form. This compound and other similar surface active agents have been described by Cornforth *et al.* (1955).

Male and female mice of the " Strong A " strain weighing 18–22 g. were used throughout this study.

Vaccination with BCG was accomplished by the intraperitoneal inoculation of 1·0 mg. of a freeze dried BCG preparation. Macrocyclon in the desired amount also was administered intraperitonally.

Mice were infected by the intravenous administration of 1·0 mg. of the H37Rv strain of *Mycobacterium tuberculosis* contained in 0·4 ml. of 0·01 M phosphate buffer pH 7·0. The cultures from which the suspensions were prepared were grown on a modified Proskauer and Beck medium (Youmans and Karlson, 1947). The harvesting of these cells, and the preparation and standardization of the suspension used for infection previously has been described in detail (Youmans and Karlson, 1947).

The technique used for enumerating the number of viable particles of tubercle bacilli in the organs of mice has been described by Sever and Youmans (1957*a*). However, several modifications of the procedure necessitate the repetition of some portions of this technique here.

At appropriate intervals following infection 5 mice were killed by crushing their skulls. The lungs and in some cases the spleens were removed aseptically and weighed. The organs then were homogenized individually by grinding for 2 min. at 110 r.p.m. with sterile Teflon grinders in matching tubes. The tissue homogenate was then diluted to a volume of 10·0 ml. with 0·01 M phosphate buffer solution pH 7·0 and the homogenization procedure was continued for 2 min. Appropriate serial dilutions of this final homogenate were made in phosphate buffer solution containing 1·0 per cent of fraction V bovine albumin.

Initially, modified Proskauer and Beck medium containing 1·5 per cent agar and 5·0 per cent sterile bovine serum was used as the plating medium. Using 0·1 ml. pipettes 10 volumes of 0·02 ml. of each dilution of each homogenate were placed approximately 2 cm. apart upon the surface of 2 plates, making 5 replicas per plate. The plates were incubated at 37° in closed containers to reduce drying of the medium. At the end of 21 days, the number of colonies in those areas which showed between 10–40 colonies were counted.

For the later experiments a modification of the soft agar medium recommended by Knox (1955) was used as an enumerating medium. In this procedure, 0·125 per cent agar was incorporated into the modified Proskauer and Beck medium and dispensed in 4·5 ml. amounts into sterile Pyrex test tubes having a diameter of approximately 18 mm. After sterilization, and before solidification of the agar, the tubes were placed in a water bath and maintained at a temperature of 50°, then 0·5 ml. of sterile bovine serum was added to each tube and mixed with the medium by gentle shaking. The tubes were removed from the water bath and 0·1 ml. volumes of each dilution of each tissue homogenate were pipetted into the tubes and mixed by gentle shaking. Ten tubes were inoculated with each dilution of each homogenate. After incubation for 3 weeks at 37°, the number of colonies in each tube which contained between 10–50 colonies was counted and recorded. Before adopting this technique for the routine enumeration of the number of viable particles of mycobacteria in tissue homogenates, it was determined that no significant difference existed between the number of viable particles detected by this method and the number detected by the plating method first employed. The soft agar medium presents several advantages. The contamination which so frequently occurs on an agar medium in Petri dishes incubated for prolonged periods was completely

eliminated. Secondly, dehydration of the medium can be more easily controlled. Lastly, counting of the number of colonies is much easier since there is no tendency for the colonies to spread and coalesce.

Following the enumeration of the number of viable particles removed from the lungs and spleens of the 5 mice the mean number of particles per organ was calculated and from these means the mean number of viable particles in the lungs or spleens of the 5 mice calculated. The confidence limits of the mean number of organisms recovered per organ and the probability of significant change in means was determined for a probability of 0·05 (Sever and Youmans, 1957*a*).

In each experiment enough mice were infected so that data could also be obtained upon the effect of the treatment or vaccination on the survival of the mice. In order to accomplish this, separate groups of untreated, treated, or vaccinated mice were observed daily and a record made of the time of death of each mouse following infection. The effect of the vaccination or treatment upon the survival of the animals was measured by calculating the median survival time according to the method recommended by Litchfield (1949). When survival of the treated or vaccinated mice was prolonged to the point that the times of death were not normally distributed the effectiveness of the treatment was measured by noting the number of mice in each group which survived 30 days or longer. The reliability and validity of these evaluation procedures have been dealt with in a previous publication (Youmans and Youmans, 1957).

RESULTS

In the first experiment each mouse was injected intraperitoneally with 25·0 mg. of macrocyclon 25 hr. before being infected intravenously with the H37Rv strain of *Myco. tuberculosis*.

TABLE I.—*Number of Viable Particles of Mycobacterium Tuberculosis Found in the Lungs of Normal and Macrocyclon-treated* Mice*

Days after infection	Normal mice		Macrocyclon mice	
	Number of viable particles†	95 per cent confidence limits	Number of viable particles†	95 per cent confidence limits
1	20,220	±6,112	6,230	±3,802
7	75,040	±3,034	40,040	±5,626
14	4,089,000	±188,200	53,200	±4,992
21	—	—	46,910	±12,150
28	—	—	23,020	±12,750

* Macrocyclon administered one day before mice were infected.

† Actual number of particles = figure in Table × 10^3.

Median survival time of 20 similar control mice = 15·5 (14·0–17·0).

Median survival time of 20 similar macrocyclon treated mice not calculated since 75·0 per cent of animals survived longer than 30 days.

Table I shows the average number of viable particles recovered from the lungs of untreated and from the lungs of macrocyclon treated mice. Fig. 1 is a graphic presentation of the same data and Fig. 2 shows the mortality data acquired from equivalent groups of control and macrocyclon treated mice.

While these findings confirm the original observation of Cornforth, Hart, Rees and Stock (1951) and also those of Solotorovsky and Gregory (1952) in respect to the marked suppressive effect on experimental murine tuberculosis of such surface active agents they show, in addition, that the suppressive effect of the macrocyclon did not become evident until sometime shortly after the 7th day of the infection.

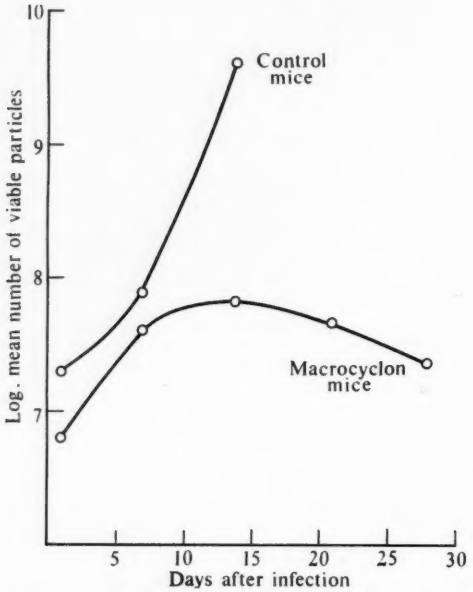

FIG. 1.—Number of viable particles of tubercle bacilli in the lungs of mice given macrocyclon one day before challenge.

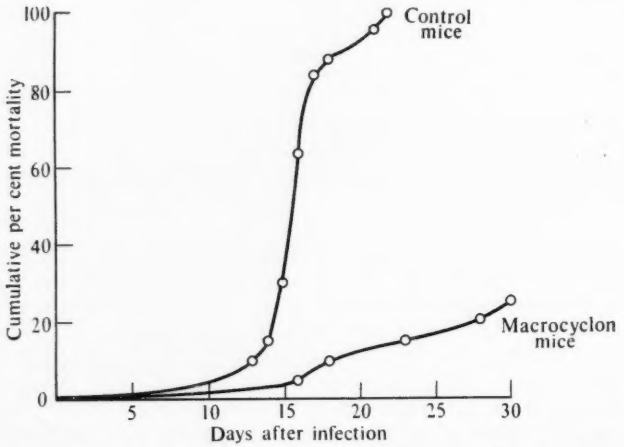

FIG. 2.—Time of death of mice given macrocyclon one day before challenge.

The second experiment was designed to determine the prophylactic effect of macrocyclon and also to compare the effectiveness of macrocyclon with the effectiveness of vaccination with an optimal dose of BCG. In this instance each mouse was given 25·0 mg. of macrocyclon 28 days prior to infection with the H37Rv strain and at the same time other mice were vaccinated each with 1·0 mg. of BCG. Suitable groups of untreated control mice also were established from the same mouse population used to form the groups of macrocyclon injected and BCG vaccinated mice. Examination of the data from this experiment as given in Table II and Fig. 3 and 4 reveal that macrocyclon even when administered 28 days prior to infection still exerted a pronounced suppressive effect on the infection. The protective effect of macrycyclon was equivalent to that of BCG.

TABLE II.—*Number of Viable Particles of Mycobacterium Tuberculosis Found in the Lungs of Normal, Macrocyclon*, and BCG Vaccinated Mice*

	Normal		Macrocyclon		BCG	
Days	Number of viable particles†	95 per cent confidence limits	Number of viable particles†	95 per cent confidence limits	Number of viable particles†	95 per cent confidence limits
1	5,834	±1,674	3,476	±1,696	5,125	±1,130
7	22,750	±2,880	36,300	±11,880	49,300	±2,660
14	779,000	±37,000	66,700	±5,880	39,200	±5,680
21	—	—	99,900	±18,040	67,840	±4,040

† Actual number of viable particles = figure in Table × 10^3.
* Macrocyclon administered 28 days before mice were infected.
Median survival time of 20 similar control mice = 15·0 (14·4–15·7).
Median survival time of 20 similar macrocyclon treated mice not calculated since 55·5 per cent survived longer than 30 days.
Median survival of 20 similar BCG treated mice not calculated since 55·5 per cent survived longer than 30 days.

Furthermore, as in the first experiment, the initial rate of multiplication of the virulent tubercle bacilli in the macrocyclon treated mice did not differ significantly from the rate of multiplication in the control, nor did it differ significantly from the rate of multiplication of the tubercle bacilli in BCG vaccinated animals. Again, some time between the first and second week, the rate of multiplication of the tubercle bacilli in the lungs of the macrocyclon treated animals and in the lungs of the BCG treated animals decreased markedly as compared with the control.

The third experiment was designed to confirm the findings of the first two and in addition to detect, if possible, whether macrocyclon administered prophylactically would also effect the mycobacterial population in the spleen.

Mice were grouped as before and to one group 25·0 mg. of macrocyclon was administered intraperitoneally. After 28 days these mice together with control mice derived from the same population as the macrocyclon mice were inoculated intravenously with the virulent H37Rv strain.

In contrast to the previous experiments, the effect of macrocyclon on the infection was not nearly so marked (Fig. 5) since all of the macrocyclon treated mice in the groups of animals used to measure survival time died in less than 30 days. There was, however, a significant difference in the median survival time between the controls and the macrocyclon animals (Table III).

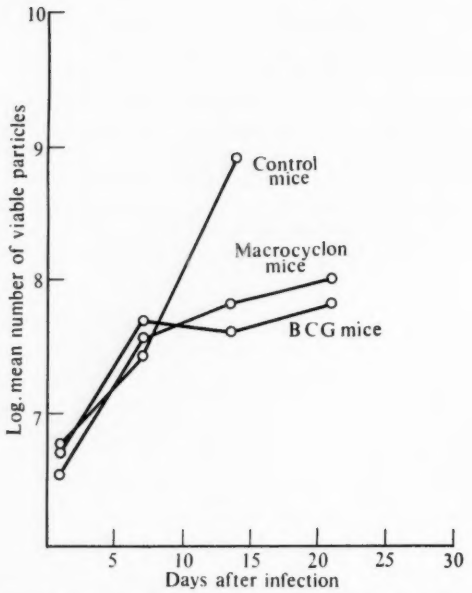

FIG. 3.—Number of viable particles of tubercle bacilli in the lungs of mice given macrocyclon 28 days before challenge and in the lungs of mice vaccinated with BCG.

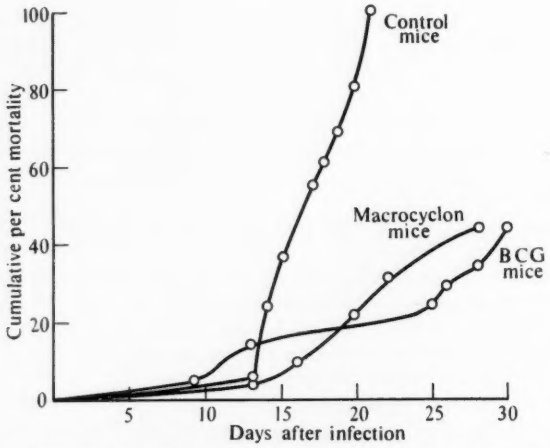

FIG. 4.—Time of death of mice given macrocyclon 28 days before challenge and time of death of mice vaccinated with BCG.

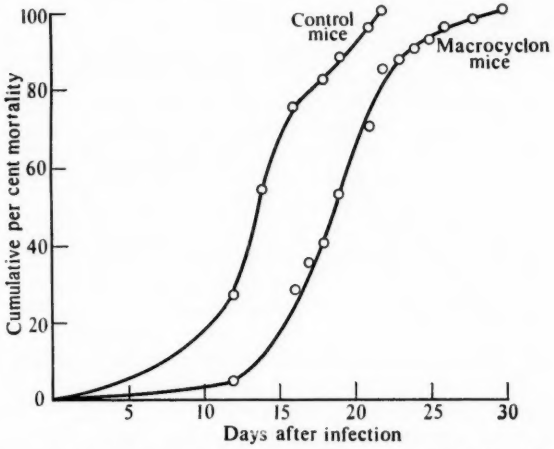

FIG. 5.—Time of death of mice given macrocyclon 28 days before challenge. (second experiment).

The enumeration data show that even though the macrocyclon effect was much less than in previous experiments the same pattern of multiplication occurred as in the 2 previous experiments (Table III, Fig. 6 and 7). Initially, in both the lungs and the spleen of the macrocyclon treated mice the tubercle bacilli multiplied at the same rate as in the control mice, then, in the spleens after 1 week, and in the lungs after 2 weeks, there was appreciable inhibition of multiplication. Enough animals survived in this group to permit enumeration of the number of viable particles at 36 and 43 days after infection. These animals showed appreciably fewer tubercle bacilli in the lungs. There was less decrease in numbers in the spleen.

TABLE III.—*Number of Viable Particles of Mycobacterium Tuberculosis Found in the Lungs and Spleens of Normal and Macrocyclon* Treated Mice*

Days after infection	Lungs				Spleen			
	Normal		Macrocyclon		Normal		Macrocyclon	
	Number of viable particles†	95 per cent confidence limits	Number of viable particles†	95 per cent confidence limits	Number of viable particles†	95 per cent confidence limits	Number of viable particles†	95 per cent confidence limits
1	23,000	±7,480	20,400	±13,860	6,200	±16,620	8,050	±4,900
8	154,000	±19,386	25,080	±10,580	28,020	±9,360	25,940	±6,000
15	8,900,000	±624,000	1,856,000	±778,000	839,400	±280,000	22,400	±4,000
29	—	—	500,400	±63,220	—	—	12,240	±2,520
36	—	—	478,600	±76,200	—	—	19,300	±4,900
43	—	—	79,160	±83,620	—	—	6,140	±32,620

† Actual number of viable particles = figure in Table × 10^3.
* Macrocyclon administered 28 days before mice were infected.
Median survival time of 20 similar control mice = 13·3 (12·0–15·0).
Median survival time of 20 similar macrocyclon treated mice = 18·5 (18·0–20·0).

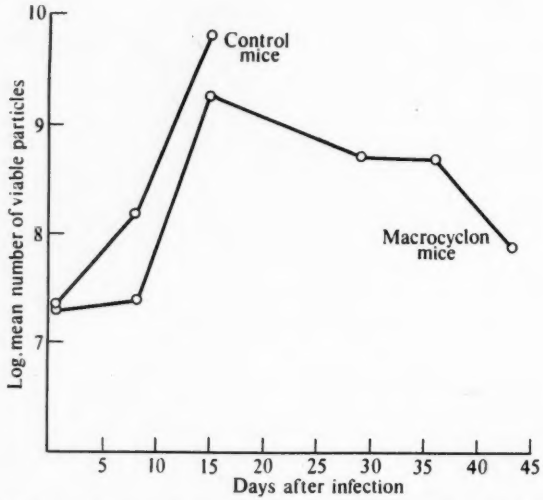

FIG. 6.—Number of viable particles of tubercle bacilli in the lungs of mice given macrocyclon 28 days before challenge (second experiment).

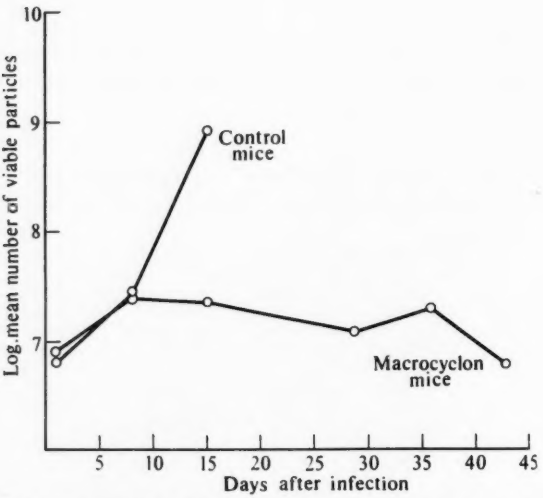

FIG. 7.—Number of viable particles of tubercle bacilli in the spleens of mice given macrocyclon 28 days before challenge (second experiment).

DISCUSSION

The results of the experiments reported in this paper confirm that macrocyclon prolongs the life of mice with acute experimental tuberculous infection. The antituberculous effect is also clearly demonstrated by counting the bacterial populations in the lungs and spleens. An unexpected finding was that in the organs of the macrocyclon treated animals multiplication of the tubercle bacilli proceeded for a period of about 1–2 weeks at a rate comparable to the rate of multiplication in untreated animals, and only then did the inhibition of multiplication become manifest—and rapidly become complete. It had been anticipated from the work of Mackaness (1954), who found that the multiplication of virulent tubercle bacilli was rapidly inhibited within macrophages taken from " Triton WR 1339 " treated animals, that inhibition of multiplication in the lungs and spleens of mice would occur shortly after infection.

The lack of inhibition of multiplication of tubercle bacilli in the macrocyclon treated mice during the first 1–2 weeks cannot be accounted for by the time that might be required for the macrocyclon to reach an effective concentration in the macrophages since this same early period of rapid multiplication was seen in the lungs of animals to whom macrocyclon was administered 28 days before they were infected as well as those to whom it was given 24 hr. before infection.

Hart and Rees (1960) also find a delay in the development of the antituberculous effect of macrocyclon, as shown by viable counts, but the results differ from ours in that some effect was already shown within the first week of infection.

Reasons for the different results obtained in the two laboratories possibly can be found among one or more of the following : Size of the infecting dose, rate of multiplication of tubercle bacilli in the respective strains of mice, degree of virulence of the H37Rv culture employed, and nature of strain of mouse.

The size of the infecting dose of a mycobacterial culture can exert a profound effect on the *in vivo* action of mycobacteriostatic drugs, and, since Hart and Rees employed a substantially smaller infecting inoculum, the earlier effectiveness of macrocyclon noted in their experiments might be accounted for on this basis alone.

Calculations using the data provided by both groups of investigators show that the rates of multiplication of the tubercle bacilli were the same in the control animals of both strains of mice. This confirms our previous observation (Sever and Youmans, 1957*b*) to the effect that in non-immune mice the rate of multiplication of the H37Rv strain was the same regardless of the size of the infecting dose, and did not differ significantly between three mose strains. Therefore it is apparent that differences in rate of multiplication *in vivo* cannot account for the discrepancies, nor do the strains of H37Rv appear to differ in virulence.

In contrast to the similarity noted between the response of mouse strains following initial contact with virulent tubercle bacilli, recent studies have shown that mouse strains may vary markedly in their immunological response to mycobacterial infection (Youmans, Youmans and Kanai, 1959). Some strains of mice develop acquired immunity much more rapidly and to a much higher degree than other mouse strains. The Strong A mouse strain immunizes moderately well, and, following administration of small infecting doses, acquired immunity will appear about 14 days after infection (Sever and Youmans, 1957*b*) since cessation of multiplication of tubercle bacilli in the tissues will occur about this time. The data of Hart and Rees show, on the other hand, that the tubercle bacilli multiplied

exponentially for a period of at least 21 days in the lungs of their macrocyclon treated animals. By 28 days multiplication had ceased, presumably due to the forces of acquired immunity. It appears that the 2 strains of mice may differ appreciably either in the rapidity of the immune response to tuberculous infection or in the amount of immunity produced.

It follows from the above considerations that the delay in the development of the antituberculous effect of macrocyclon conceivably could be accounted for by the appreciably larger infecting inoculum employed in our study, and, the earlier almost complete cessation of multiplication could have resulted from a more rapid or greater immune response in the Strong A mouse strain.

A comparison of the changes in the microbial population in the lungs of the macrocyclon treated animals with the changes in the microbial population of the lungs of the BCG treated animals is of interest. The similarity between these 2 groups may be coincidental, since we have shown previously that in more highly immunized animals inhibition of multiplication of tubercle bacilli may occur very soon after inoculation (Sever and Youmans, 1957*b*). The data do show, however, that the same sudden cessation of multiplication of tubercle bacilli may occur, and at approximately the same time, in partically immunized mice as occurs in the macrocyclon treated mice. It is possible, indeed, that macrocyclon may exert its antituberculous effect by either directly or indirectly stimulating the forces of acquired immunity.

Finally, it is of interest to note that the time at which suppression of growth is noted in both the macrocyclon treated and BCG treated animals corresponds to that time after infection at which in untreated mice an extracellular phase of the infection begins to develop. This occurs in mice injected with numbers of tubercle bacilli comparable to the ones used in this experiment—because of the rupture of macrophages from the accumulation, due to multiplication, of mycobacteria within the macrophage cytoplasm (Nyka, Faherty, Malone and Kiser, 1954). Fong, Schneider, and Elberg (1956, 1957) have shown *in vitro* that macrophages obtained from immunized animals are less susceptible to the destructive effect of intracellular virulent mycobacteria than are macrophages obtained from normal animals, and, more recently, Patnode and Hudgins (1959) have demonstrated that macrophages from BCG immunized animals are appreciably more resistant to disruption by sonic vibration than are macrophages from normal animals. This increased resistance to destruction might account, at least in part, for the eventual suppression of multiplication of tubercle bacilli which occurs in the second week of infection in the macrophages of immunized animals. Hudgins and Patnode (1958) have shown that macrophages from animals treated with Triton WR 1339 are also more resistant to destruction by sonic vibration. Lovelock and Rees (1955) have demonstrated that macrocyclon will increase the resistance of red cells to thermal shock and the work of Mackaness (1954) indicates that macrophages obtained from " Triton A–20 " treated animals survive longer when infected with tubercle bacilli than do macrophages obtained from normal animals.

SUMMARY

Macrocyclon administered to mice 28 days prior to challenge exerted a suppressive effect on an experimental tuberculous infection although the suppressive activity was less than when adminstered 1 day before challenge.

The number of viable particles of virulent tubercle bacilli in the lungs of normal mice, mice vaccinated with BCG, and mice treated with macrocyclon, was determined at weekly intervals following intravenous challenge. The data revealed that in all three groups of animals the multiplication of tubercle bacilli in the lungs proceeded at the same rate for a period longer than 7 days. However, at some time between the 7th and 14th day multiplication of the tubercle bacilli was rapidly brought to a halt in the lungs of the BCG vaccinated and the macrocyclon treated animals, whereas multiplication continued unchecked in the lungs of the untreated control mice. A similar pattern of suppression of multiplication of tubercle bacilli was found in the spleens of macrocyclon treated mice.

Possible mechanisms whereby a surface active agent such as macrocyclon might bring about a pattern of suppression of multiplication of tubercle bacilli *in vivo* almost identical with that produced by the forces of specific acquired resistance have been discussed.

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EFFECT OF MACROCYCLON IN ACUTE AND CHRONIC PULMONARY TUBERCULOUS INFECTION IN MICE AS SHOWN BY VIABLE AND TOTAL BACTERIAL COUNTS

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ANTITUBERCULOUS drugs are generally assessed by their ability to prolong life or reduce lesions in animals with acute tuberculosis. The trends of the viable populations of tubercle bacilli in the lungs and spleens of mice have also been made the basis of assessment of various drugs, a chronic tuberculous infection being used (McCune and Tompsett, 1956 ; McCune, Tompsett and McDermott, 1956). We have extended this technique by following in parallel the viable and the total (stainable) bacillary population in the lungs of mice with acute and chronic tuberculous infection. The object of such dual counts was to distinguish a bacteriostatic from a bactericidal action.

The method of dual counts has been used previously for untreated infected mice, but fell into disrepute since the total count was often inexplicably less than the viable (see Gray and Jennings, 1955). However, Fenner (1951) showed that, with careful examination of suspensions of tubercle bacilli from tween 80-containing culture medium, the number of stained organisms was not significantly different from the viable count. Affleck and Gray (1957) had similar results with counts from lungs of mice with slowly progressive tuberculous infection. We have also found close correspondence betwen total and viable counts from mouse lungs in untreated acute and chronic infections. Apparently the method has not previously been used for analysing the effect of drugs on tuberculous infections.

Macrocyclon is a surface-active agent with suppressive effect on acute experimental tuberculous infections in mice and guinea-pigs, but without antituberculous effect *in vitro* (Cornforth, Hart, Rees and Stock, 1951 ; Cornforth, Hart, Nicholls, Rees and Stock, 1955 ; Rees, 1953, 1958). The dual method of population counts seemed a promising method for analysing the effect of this unusual drug *in vivo*. It seemed of particular interest to study the effect, if any, of macrocyclon in chronic murine tuberculosis, an infection resembling chronic pulmonary tuberculosis in man.

Our approach to the problem of macrocyclon is complementary to that of Niffenegger and Youmans (1960).

MATERIALS AND METHODS

Acute infection.—Female mice (18–20 g.) of the albino P strain were infected intravenously with 0·1 mg. wet-weight bacilli (containing about 4×10^7 viable units) of the human virulent strain of *Mycobacterium tuberculosis* H37Rv, prepared by grinding a fully-grown culture (2–3 weeks old) obtained from the surface of Proskauer and Beck's liquid medium. Under these conditions the median survival time is usually 18–21 days, with all dead by 30–35 days and showing gross pulmonary tuberculosis.

Chronic infection.—As above, but the infecting dose was reduced to 0·02 mg. wet-weight bacilli. Only about 30 per cent of the mice died from tuberculosis within the first 8 weeks (80 per cent of these deaths, in fact, occurred by the 5th week). The majority of the remaining mice survived in an apparently healthy state for many weeks with an established and predominantly pulmonary infection. Mice killed during this stable phase showed discrete and proliferative-type lesions in the lungs. During the stable phase a few mice die of respiratory insufficiency (with obvious cyanosis) due to cellular infiltration and oedema of the lungs and not due to progression of the infection with an increase in the number of tubercle bacilli (Sever and Youmans, 1957). This fairly stable infection is similar to the one used for studying the effect of cortisone and of suramin on chronic mouse tuberculosis (Hart and Rees, 1950; Rees and Hart, 1956) produced with an infecting dose of only 0·002 mg. bacilli. A larger infecting dose was chosen for the present study in order to obtain an adequate number of tubercle bacilli in the lung for accurate counting.

Macrocyclon treatment.—Macrocyclon, a polyethylene glycol ether of a *p-tert*-octylphenol-formaldehyde cyclic tetramer (with average 12½ ethylene oxide units per phenolic group), was autoclaved as a 12·5 per cent solution in 0·45 per cent saline, and 25 mg. in 0·2 ml. injected subcutaneously twice weekly.

For the acute infections the treatment was started 2 days after the infection. For the chronic infections it was started 60 days after the infection.

Preparation of lung homogenates.—At appropriate intervals 3–4 mice were taken at random from apparently healthy members of the treated and/or untreated group, in the case of chronic infections; and from the fittest members of the control group and from any of the macrocyclon group (because they were all fit), in the case of acute infections. They were killed by exposure to coal-gas, and under sterile conditions the chest wall was opened and both lungs removed. The lungs from each mouse were homogenized individually under aseptic conditions by hand with pestle and mortar, finally suspending each lung in 10 ml. 0·01 M phosphate buffer pH 7·0.

Number of viable units of tubercle bacilli in lungs.—Viable counts were made by a modification of the method of Miles and Misra (1938). Suitable dilutions (in 0·1 per cent albumin-saline) of each homogenate were made within an hour, and 0·02 ml. drops were inoculated on plates of solid nutrient medium. The medium was oleic acid-albumin agar (Dubos and Middlebrook, 1947), modified according to Fenner, Martin and Pierce (1949) and (in the method of incorporating the glucose) to Yegian and Budd (1951). Counts were made after incubating the plates at 37° for 14 days in plastic bags.

Total number of acid-fast bacillary units in lungs.—Total bacillary counts (of stainable acid-fast bacilli) were made concurrently with the viable counts on each homogenate of mouse lung by a modification of the method described by Hobby, Hanks, Donikan and Backerman (1954). It is the method used in these laboratories for counting *Mycobacterium lepraemurium*, referred to by Rees and Wong (1958) but not previously described in detail. An 0·003 ml. drop of suitable dilutions (in 0·1 per cent albumin–1·6 per cent formaldehyde-water) of each lung homogenate was spread over an 8 mm. diameter circle marked out on a glass slide (4 circles per slide). Spread-smears were prepared in quadruplicate from each dilution. The albumin/formalin diluting fluid acted as both a protein-filming and a fixative agent. The spread-smears were finally fixed on the slide by drying on a lamp housing for 5 min., exposing to formalin fumes for 5 min., and reheating, to drive off the formalin, for a further 5 min. The smears were stained by the Ziehl-Neelsen method, modified to avoid loss of bacilli: carbol fuchsin for 2 min.; gentle washing in water and decolorizing and counter-staining in one process, using a mixture of 4 per cent sulphuric acid in 0·2 per cent methylene blue for 1 min.

Stained bacilli were counted in 16 microscope fields per smear, using a 2 mm. oil immersion objective and × 6 binocular eyepieces. The fields were 1 mm. apart, 8 along each of 2 diameters, set at right angles. Four spread-smears were counted from each dilution. The average number of bacilli/microscope field was determined, and the number of bacilli/lung calculated by the use of calibration factors for the optical system, the area of the smear and the dilution factor. The spread-smears were almost cell-free and the bacilli either single or in small groups. Since a " viable unit " count is based on the assumption that a colony can originate from a single bacillus or group of bacilli, the total bacillary counts in this study were brought into line by counting each stained bacillus or group of bacilli as one unit—giving the " total acid-fast bacillary unit count."

All counts were expressed as $\log_{10}$ viable, or total acid-fast bacillary, units/organ. Simple averages of these logarithms were used for the charts.

RESULTS

Effect of macrocyclon in acute tuberculosis

Thirty-six mice were infected and randomized into 2 equal groups, untreated and macrocyclon-treated (see p. 00). Treatment with macrocyclon was started on day 2 and was continued twice weekly until day 26. Mice were killed on days

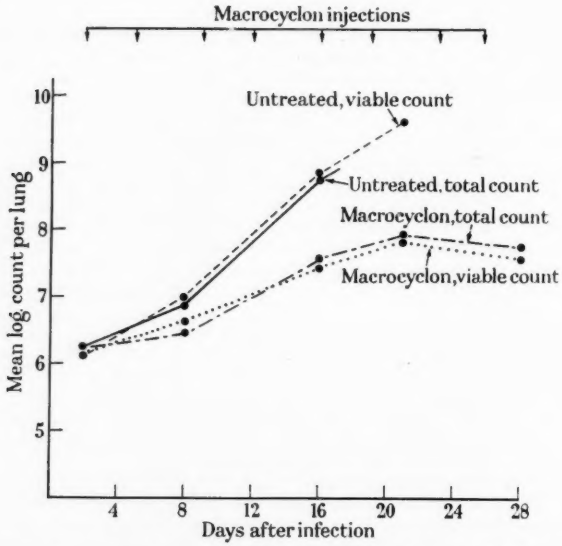

FIG. 1.—Influence of macrocyclon on viable and total populations of *Myco. tuberculosis* in mouse lungs during an acute infection (0·1 mg. H37Rv).

There were too many bacilli in the homogenate from untreated mice on day 21 for an accurate total log count ($> 9{\cdot}2$).

2, 8, 16, 21 and 28. On day 2, 4 mice were killed from the untreated group, and on day 28, 4 from the treated group ; while on the intermediate days 3 mice were killed from each group. The mice killed from the untreated group were fit except for 2 slightly sick and 3 very sick, on days 16 and 21, respectively. No untreated mice were alive on day 28. The individual and average viable and total bacillary units/lung at each time of sacrifice, expressed as $\log_{10}$, are given in Table I, and the average lung counts are also shown in Fig. 1.

Table I shows that the total count in each lung from the untreated mice was very similar to the viable count ; in 5 of the 10 paired figures the total counts slightly exceeded the viable, while in 5 they were slightly less. It may be concluded that most of the bacterial population was viable. Fig. 1 shows a continuous rise in both the viable and the total counts from the untreated mice, indicating a multiplying population.

TABLE I.—*Viable and Total Counts of Bacilli in the Lungs of Untreated and Macrocyclon-treated Mice in Acute Tuberculosis*

	Untreated					Macrocyclon-treated*				
Day	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)
2	1	6·04	6·16	6·08	6·25	—	—	—	—	—
	2	6·40		6·08		—	—	—	—	—
	3	6·15		6·38		—	—	—	—	—
	4	6·04		6·47		—	—	—	—	—
8	1	6·90	7·03	6·95	6·90	1	6·57	6·61	6·25	6·49
	2	7·10		6·89		2	6·73		6·55	
	3	7·08		6·85		3	6·54		6·68	
16	1	8·34	8·87	8·37	8·80	1	7·83	7·46	7·92	7·59
	2	9·15		9·02		2	7·59		7·87	
	3	9·13		9·01		3	6·95		6·98	
21	1	9·61	9·61	>9·2	>9·2†	1	7·95	7·87	7·97	7·92
	2	9·65		>9·2		2	8·15		8·08	
	3	9·57		>9·2		3	7·51		7·70	
28‡	—	—	—	—	—	1	7·18	7·59	7·35	7·80
	—	—	—	—	—	2	8·06		8·23	
	—	—	—	—	—	3	7·95		8·19	
	—	—	—	—	—	4	7·15		7·42	

* Treatment, subcutaneously twice weekly, started day 2 and continued to day 26 (inclusive).
† Too many bacilli in spread-smears for accurate count.
‡ All untreated mice died with acute tuberculosis before day 28.

The divergence between the counts from the macrocyclon-treated and the untreated mice (Fig. 1) indicates a definite, but not powerful, antibacterial effect, which became more effective after day 8 and even more so after day 16. The closeness of the curves for total and viable counts in the treated group indicates that macrocyclon had little or no killing effect on the tubercle bacilli, but that its action was bacteriostatic. The latter conclusion is supported by the paired individual counts (Table I), which were very similar to one another ; in 10 out of 13 the total count slightly exceeded the viable, while in 3 it was slightly less. Similar results were obtained in a second, duplicate, experiment.

Effect of macrocyclon in chronic tuberculosis

Fifty surviving mice from an original stock of 90 infected (33 per cent having died from tuberculosis) were randomized into 2 groups on day 60, untreated (27 mice) and macrocyclon-treated (23 mice). Treatment with macrocyclon was started on day 60 and continued twice weekly until day 131, representing a total of 21 doses. Mice were killed on days 60, 88, 111 and 134. On day 60, 4 mice were killed from the untreated group ; on days 88 and 111, 3 mice and on day 134, 4 mice were killed from both the untreated and the treated groups. Only fit mice were killed, and these were picked each time at random from the 2 groups. It was not considered necessary to withhold treatment just before killing, in order to avoid carry-over of macrocyclon in the lung homogenates, since there is no evidence that the drug is antituberculous *in vitro*. The individual and average viable

and total bacillary units/lung at the time of death, expressed as $\log_{10}$, are given in Table II, and the average lung counts are also shown in Fig. 2.

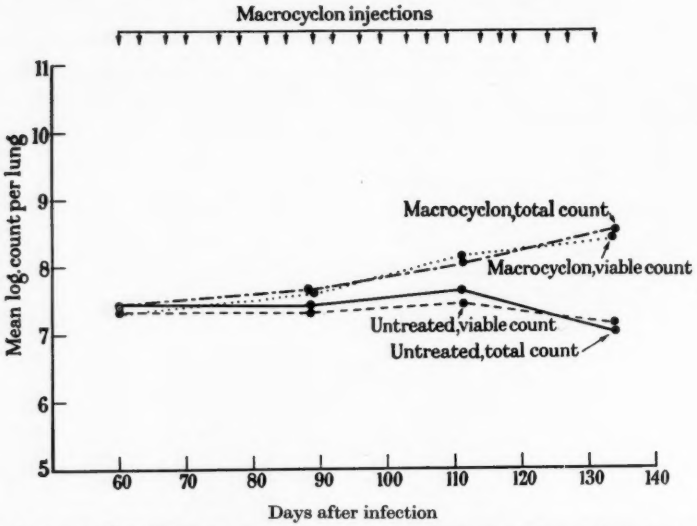

FIG. 2.—Influence of macrocyclon on viable and total populations of *Myco. tuberculosis* in mouse lungs during a chronic infection (0·02 mg. H37Rv).

TABLE II.—*Viable and Total Counts of Bacilli in the Lungs of Untreated and Macrocyclon-treated Mice in Chronic Tuberculosis*

	Untreated					Macrocyclon-treated*				
Day	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)	Mouse	Viable count (log)	Average viable count (log)	Total count (log)	Average total count (log)
60	1	6·93	7·32	6·89	7·42	—	—	—	—	—
	2	7·57		7·77		—	—	—	—	—
	3	7·59		7·83		—	—	—	—	—
	4	7·18		7·18		—	—	—	—	—
88	1	7·27	7·32	7·42	7·44	1	8·04	7·66	8·05	7·69
	2	6·88		6·98		2	7·67		7·64	
	3	7·80		7·91		3	7·26		7·38	
111	1	8·15	7·46	8·18	7·65	1	8·38	8·15	8·21	8·05
	2	7·22		7·43		2	7·04		7·12	
	3	7·02		7·34		3	9·03		8·83	
134	1	6·67	7·16	6·73	7·06	1	9·22	8·51	9·28	8·54
	2	8·53		8·38		2	8·57		8·69	
	3	6·79		6·73		3	7·53		7·44	
	4	6·66		6·38		4	8·70		8·75	

* Treatment, subcutaneously twice weekly, started day 60 and continued to day 131 (inclusive).

Table II shows that the total count in each lung from the untreated mice was very similar to, though usually slightly greater than, the viable count ; in 9 of the 14 paired figures the total counts slightly exceeded the viable, while in 5 they were slightly less. It may be concluded that there was no build-up of dead bacilli in the lungs, most of the bacterial population in this chronic tuberculous infection being viable. This table, as well as Fig. 2, shows that both viable and total counts from the untreated mice remained remarkably constant throughout the period of 74 days, indicating a steady population.

Fig. 2 also shows that, unlike the results obtained in acute tuberculosis, macrocyclon had no antibacterial effect in this chronic infection, even when treatment was continued throughout a period of 10 weeks. Bearing in mind that, without treatment, the bacterial population was steady, this finding is not inconsistent with the bacteriostatic action of macrocyclon, observed in the acute infection.

Although the results show that macrocyclon had no antibacterial effect in chronic tuberculosis, there was a tendency in this experiment for the bacillary population actually to increase in the drug-treated animals (Fig. 2). However, in a similar, though shorter, experiment, which confirmed the lack of antibacterial effect of the drug, there was no increase in the bacillary population of the treated animals above that of the untreated.

DISCUSSION

Macrocyclon is remarkable in showing no direct antibacterial effect *in vitro*. Its antituberculous effect is presumed to be due to interaction with the host defence mechanisms—possibly within macrophages, which it undoubtedly enters (Lovelock and Rees, 1955 ; Cornforth, Potts and Rees, unpublished). It is this drug-host interaction, resulting in an indirect antibacterial effect, that has been analysed in the present experiments.

The antibacterial effect of macrocyclon in acute murine tuberculosis is attributable, from the closeness of the total and viable counts, to a bacteriostatic rather than bactericidal action. In the chronic infection, on the other hand, the drug was not antibacterial. Since the population of the untreated controls in the latter infection was steady throughout the experiment, and the viable and total counts remained close to one another, it may be inferred that, unless dead organisms were being removed, the majority of the bacilli were not multiplying and were therefore in a comparatively static or resting phase. The contrast between the effectiveness of macrocyclon *in vivo* against a freely multiplying population, as found in the acute infection, and its ineffectiveness against a non-multiplying population, represented by the chronic infection, is analogous to similar contrasts *in vitro* with a number of other drugs (Singh and Mitchison, 1955 ; Mitchison and Selkon, 1956 ; Hobby and Lenert, 1957).

The ineffectiveness of macrocyclon in the chronic murine infection is strikingly different from that of the very powerful combination, pyrazinamide-isoniazid. This has been shown to be markedly antibacterial in this type of mouse infection (McCune, Tompsett and McDermott, 1956) ; moreover, the lung viable count falls rapidly below the total count (Rees and Hart, unpublished).

In the preceding paper Niffenegger and Youmans (1960) also report an antituberculous effect of macrocyclon in acute murine infection, using viable population counts. The early trends of these counts following infection in treated and

untreated mice were somewhat different from those observed in our experiments, and they discuss these differences. Macrocyclon was shown by these authors to be active even when administered prophylactically 28 days before an acute infection. We obtained similar prophylactic effects in acute tuberculosis, judged by survival. When a single dose of macrocyclon was given 1, 2 or 3 weeks before an acute infection, it was as effective as a dose given one day after ; but the effect was less at 4 weeks and unobtainable at 5 weeks before the infection (Rees, unpublished). Niffenegger and Youmans suggest that macrocyclon may affect the host immune response in a similar manner to BCG. If this be so, the inactivity of macrocyclon in our experiments on chronic murine tuberculosis might result from the immune responses being already fully stimulated by the established infection itself. This explanation would be an alternative to that suggested above (ineffectiveness of the drug against a resting population).

Recently a pilot clinical trial of macrocyclon has been carried out in patients with advanced chronic pulmonary tuberculosis (Boyd, Stewart, Somner, Crofton and Rees, 1959) ; but the drug was ineffective, although this type of chronic disease can respond to other forms of chemotherapy. It is obviously tempting to suggest chronicity of the infection as the common causative factor for the ineffectiveness of macrocyclon in chronic disease in man and mouse. The tubercle bacilli in both instances would generally be either in a resting phase or multiplying slowly, compared with the rapid rate of multiplication in acute murine infections. Macrocyclon can, however, have a therapeutic effect in man, as has been shown by Dr. J. A. McFadzean in a pilot trial of the drug in an infection with another mycobacterial species—leprosy.

SUMMARY

The effect of macrocyclon, a surface-active agent that is antituberculous *in vivo* but not *in vitro*, was studied in tuberculous mice by dual bacterial counts—viable and total (stainable)—in lung homogenates. Two types of experimental infection were used : acute and fatal produced by a large dose of bacilli, and chronic, following a smaller dose. The drug was given subcutaneously twice weekly, from 2 until 26 days after the acute infection and from 60 until 131 days after the chronic infection.

In the acute infection the total counts from the lungs of untreated mice were very similar to the viable counts throughout, indicating that most of the bacterial population was viable ; and the continuous rise in both counts showed that the population was multiplying. The counts from the treated mice diverged from these counts, indicating a definite though not powerful antibacterial effect, which was more manifest after the first week and even more so after a fortnight. From the close similarity of the total and viable counts in the treated group it was concluded that macrocylon's action was bacteriostatic rather than bactericidal.

In the chronic infection the two counts from the untreated mice were also very similar, indicating that here too most of the bacterial population was viable, without a build-up of dead bacilli in the lungs ; but (unlike the acute infection) both counts remained constant throughout the observation period, indicating a steady population. The trend of the counts from the treated mice showed no evidence of any antibacterial effect by macrocyclon. It was considered that, unless dead organisms were being removed from the lungs of the untreated

animals, the majority of the bacilli were not multiplying and that in this resting phase they may not have been susceptible to the drug's action.

We are indebted to our colleague Dr. J. W. Cornforth, and to Imperial Che-Chemical Industries (Pharmaceuticals) Ltd. for the macrocyclon; to Dr. W. Steenken, Jr., for the H37Rv strain of *Myco. tuberculosis*; and to Mr. C. V. Gibbs for his technical assistance.

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IDENTIFICATION OF ACID MUCOPOLYSACCHARIDES FROM GRANULATION TISSUE IN RATS*

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THE acid mucopolysaccharides (MPS) of "ground substance" presumably play an important role in inflammation. As the burden of repair associated with the inflammatory reaction falls largely upon connective tissue, the tissue thus formed may be expected to contain different chemical units of connective tissue including more than one MPS, as found in many connective tissues (Meyer, Davidson, Linker and Hoffman, 1956). Most information concerning MPS with inflammatory changes has been gained by techniques of histochemistry, and some by observations of isotope incorporation into the MPS, but the actual MPS that are involved in inflammation have not been identified. Although the physiological implications of the MPS, as a group or individually, are not clear at this time, identification of the chemically different compounds which take part in inflammation and repair seem to be important. In the following experiments 4 distinct MPS were isolated and identified from inflammatory tissue.

The methods of injecting air and an irritant, as employed by Selye (1953), and of using irritants alone by others (Menkin, 1950; Taubenhaus and Amromin, 1949) to produce granulation tissue have been used in many experiments to study inflammation. The air-granuloma pouches are easily produced, consist of large amounts of tissue, and can be obtained as discrete units, separate from surrounding connective tissue. This model was therefore selected to study the type of MPS which are produced by an inflammatory response.

MATERIALS AND METHODS

Production of inflammatory tissue.—Rats weighing 180–250 g. from a Long-Evans strain were given standard laboratory diet and water freely. Granuloma pouches were produced by injection of 20–30 ml. of air and turpentine 0·05 ml./10 g. body weight into the subcutaneous tissue of the back.

In preliminary experiments it was observed that the maximum amount of tissue was formed between the 7th and 10th days at which time the lesions could be dissected easily from the skin and surrounding subcutaneous tissue. After the turpentine was blotted or drained, the granulomata were obtained in yields of approximately 5·0 g./animal. Granulomata were obtained from approximately 250 rats, an amount of tissue adequate to isolate and identify the MPS. Seven- and 10-day-old lesions were studied in similar fashion.

One study was performed to observe variations of the granuloma tissue with time and will be described in detail later.

Isolation of mucopolysaccharides.—The MPS were isolated by methods that have been described previously for studies of other tissues (Schiller, Mathews, Jefferson, Ludoweig and Dorfman, 1954; Berenson, 1958). The procedure briefly consists of mincing the granuloma

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tissue, defatting with acetone and air drying. The dry-defatted tissue, approximately 15 per cent of wet weight, was extracted with alkali, hydrolyzed with trypsin and treated with trichloroacetic acid for deproteinization, and the MPS were precipitated with alcohol. The initial preparation, 8·0 (4·6–14·0) mg./g. dry-defatted granuloma, thus isolated is a crude mixture of MPS which analysed 3·4 per cent N, 7·1 per cent hexosamine and 10·6 per cent uronic acid. The mixture was fractionated by zone electrophoresis and by 2 column chromatographic methods (Berenson, Roseman and Dorfman, 1955 ; Berenson, 1958).

Analyses of isolated compounds.—A number of analyses were performed to establish the identity of the isolated compounds. The following were calculated on the basis of dry weight obtained at 105° : (1) optical rotation, (2) nitrogen by micro-Kjeldahl digestion and nesslerization, (3) uronic acid (Dische, 1947), (4) hexosamine (Elson and Morgan, 1933), and (5) sulphate (Toennies and Bakay, 1953). The effect of enzymatic hydrolysis with testicular hyaluronidase (Dorfman and Ott, 1948 ; Mathews, Roseman and Dorfman, 1951) was observed, and identification of hexosamine (Stoffyn and Jeanloz, 1954) and uronic acid (Fischer and Dörfel, 1955) was achieved by paper chromatography. Infra-red spectral analysis was used to aid in identification of chondroitin sulphate.

RESULTS

The results are summarized in Tables I and II and in Fig. 1–3.

Time-course production of granuloma tissue

Variations in the granuloma tissue were studied serially in 60 male rats. At intervals of 3, 5, 7, 10 and 14 days after preparation of the animals, groups of 10 were randomly selected and killed. The inflammatory tissue from each group was analysed for yield, content of water, N and hexosamine. The results are shown in Table I.

TABLE I.—*Time-course Production of Granuloma Tissue*

Age of granuloma (days)	Granulation tissue (g./animal)	H_2O (per cent)	N (per cent)	Hexosamine (per cent)
3	2·87	72·8	8·3	0·66
5	3·11	77·8	8·0	0·75
7*	3·00	79·8	8·7	0·82
	5·5 (4·7–6·2)	76·8 (75–79·3)	—	—
10*	1·81	79·1	8·2	0·73
	4·9 (2·5–7·5)	77 (67–85·8)	9·0 (4·2–13·2)	0·63 (0·35–0·95)
14	0·95	80·4	9·6	0·76

* The average values and ranges obtained from a number of experiments (approximately 20) are shown on the lower lines.

As has been described in earlier studies (Selye, 1953), a progressive change in character and appearance of the granuloma pouches occurs over a period of 14 days. Detailed, carefully performed histological studies of serial changes in experimentally-induced granuloma tissue can be found in the works of others (Selye, 1953 ; Williams, 1957). Obvious is the variation in the total weight of the granuloma, and as organization of the lesion progresses with formation of fibrous tissue, the weight begins to diminish. The last period of observation, 2 weeks after formation of the pouches, just precedes erosion of the skin and expulsion of the contents to the outside. No consistent change in content of water, N, or hexosamine was observed, although similar investigations with various irritants—turpentine, croton oil, cellulose sponge, or carrageenin (Layton, 1950 ;

TABLE II.—*Analyses of Mucopolysaccharides Isolated by Zone Electrophoresis from Granulation Tissue*

Age of granuloma (days)	MPS*	Amount isolated (mg./g. dry-defatted granuloma)	Optical rotation ($[\alpha]_D^{23}$)	N (per cent)	Hexosamine (per cent)	Hexuronic acid (per cent)	S (ester sulphate) (per cent)	Testicular hyaluronidase hydrolysis
7	HA fraction	0·95	−38	5·1	35·1 (glucosamine)	30·5	<0·2	++
	CSA	0·54	−43	3·0	27·9 (galactosamine)	17·9†	5·3	+
10	HA fraction	0·92	−36	5·3	34·5 (glucosamine)	32·8	<0·2	++
	CSA	0·73	−38	3·4	25·1 (galactosamine)	19·3†	5·2	+

* Theory :		$[\alpha]_D^{23}$	N (per cent)	Hexosamine (per cent)	Uronic acid (per cent)	S (per cent)
	HA	−70	3·5	44	48	—
	CSA	−30	2·8	35	38	6·4

† When determined by the carbazole method, the presence of L-iduronic acid of chondroitin sulphate B accounts for the low values.

Robertson and Schwartz, 1953 ; Kodicek and Loewi, 1955 ; Boucek and Noble, 1955 ; Slack, 1957, 1958 ; Jackson, 1957, 1958)—or the technique of inducing wounds (Hudack, Blunt, Higbee and Kearin, 1949) indicated other chemical changes within the granulation tissue with time. Studies of hexosamine (Shetlar, Lacefield, White and Schilling, 1959) and hydroxyproline contents (Kao, Boucek and Noble, 1957 ; Jacob and Houck 1958) indicate striking increases in relative concentrations of hexosamine before 72 hr. For comparison, collagen formation, as indicated by hydroxyproline determinations, or by incorporation of labelled amino acids, occurs more slowly than formation of hexosamine-containing substances. Experiments with sulphate ^{35}S incorporation, to reflect metabolism of sulphated mucopolysaccharides, indicate maximum incorporation of organic sulphate by approximately one week. Although such studies are suggestive of the changes occurring in MPS in the inflammatory tissue, it might be pointed out that the determinations of hexosamine or of ^{35}S represent composite analyses of a group of compounds. Since hexosamines are uniformly a part of, and sulphate is contained in a number of MPS, their determination in a tissue does not necessarily reflect changes of an individual MPS. Since this is the case, further study of the time-course changes in inflammatory tissue of individual MPS, of the larger units, may be in order when better techniques to isolate and identify the MPS are developed.

Fractionation and identification of mucopolysaccharides

Further elimination of protein and separation of MPS into 2 groups were performed by zone electrophoresis with celite (Johns-Manville, Analytical Filter Aid) in a phosphate buffer, pH 6·9 (Fig. 1). Material from each group was pooled from several electrophoretic procedures and subjected to further electrophoresis as an additional aid to separation of the 2 groups. Analyses of these compounds

isolated from 7- and 10-day-old lesions are shown in Table II. The analyses, although similar to those of hyaluronic acid and chondroitin sulphate A, deviate from theory and were considered as mixtures of MPS. Electrophoretic separation has been shown to separate the MPS incompletely (Berenson, 1958) and further fractionations were attempted by chromatography.

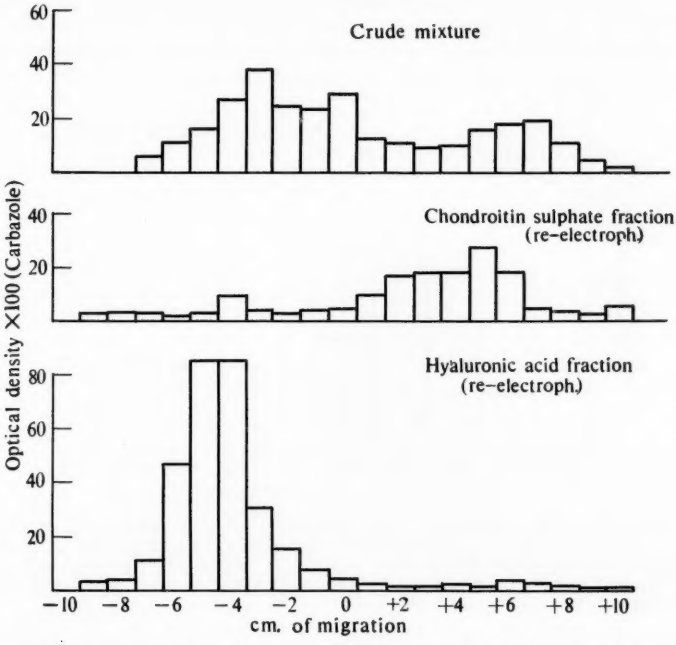

FIG. 1.—Zone electrophoresis on celite slabs of the acid mucopolysaccharides isolated from air–turpentine granuloma tissue of rats. The top graph represents the electrophoretic separation of the mixture of compounds into essentially two components. Pooled samples from a number of slabs of each component were re-electrophoresed for additional purification before fractionation by chromatography, illustrated by the other graphs. Slight deviations from the centre of the three procedures shown are due to mechanical variations. The analyses of the fractions isolated by electrophoresis are shown in Table II.

The material with positive migration demonstrated similar mobility for known chondroitin sulphate in this electrophoretic system. After isolation it was chromatographed on columns of celite No. 545-cellulose paper and fractionated with 0·1 M solutions of zinc acetate and propanol. Two components could be separated, as shown in Fig. 2. Studies by paper chromatography indicated the hexosamine of both substances to be galactosamine. The major compound, representing about two-thirds, demonstrated an optical rotation of $[-33]_D^{23}$ and equimolar quantities of glucuronic acid, hexosamine and sulphate. It was hydrolyzed by testicular hyaluronidase, and the infra-red absorption spectrum was consistent with the pattern of chondroitin sulphate A. The second substance

was found to have a rotation of $[-47]_D^{23}$, equimolar hexosamine and sulphate and 0·6 moles of uronic acid by the carbazole method. This compound was resistant to digestion by testicular hyaluronidase and was found to contain L-iduronic acid by paper chromatography. The analyses indicated the second MPS to be chondroitin sulphate B.

The material isolated by electrophoresis with resultant negative migration was further divided by column chromatography on siliconized celite incorporating butanol and lauryl amine and fractionated by increasing concentrations of NaCl solutions (Fig. 3). (The negative migration is due to electro-osmosis of the buffer.

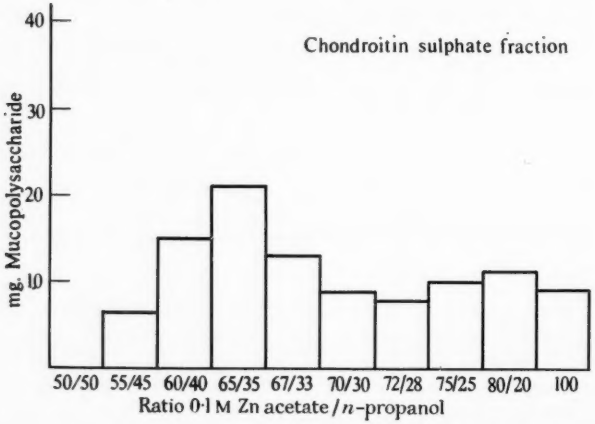

FIG. 2.—Chromatographic separation of the chondroitin sulphate obtained by zone electrophoresis. Electrophoretically uniform material isolated as chondroitin sulphate was fractionated by chromatography into two compounds. The major compound upon analysis, described in the text, was indicated to be chondroitin sulphate A and the other compound was chondroitin sulphate B (β heparin).

The resulting mobility is similar to other samples isolated as hyaluronic acid. Experiments which have been reported subsequent to completion of this work (Schiller, 1959) demonstrated the overlapping and a slightly more rapid mobility of heparitin sulphate). Two glucosamine-containing components were obtained. Approximately 80 per cent of the material was found to have an optical rotation of $[-68]_D^{23}$, equimolar uronic acid and hexosamine and was hydrolyzed by hyaluronidase; these analyses are consistent with those of hyaluronic acid. The remaining fraction analysed $[+26]_D^{23}$ with equimolar uronic acid and hexosamine, 1·2 per cent S (as ester sulphate), and was resistant to hydrolysis by testicular hyaluronidase. This fraction was considered to be heparitin sulphate.

DISCUSSION

The processes of inflammation and repair, fundamental defence mechanisms of the body, are basic in the study of disease. Both represent fascinating changes which tissues are capable of undergoing in response to injury. A number of approaches have been employed in an effort to understand more about the nature

of wound healing. Observations have been made of vascular phenomena, morphology, and cellular mechanics; more recently, interest has centred on biochemical studies of inflammatory tissue. Chemical study of even an experimental model of inflammation is interesting and may be useful to reflect both on genuine disease and on healing by connective tissue.

Although patterns of inflammation can vary with different causes or sites of injury, the process of healing of tissue is similar. Repair even in the more subtle "wear and tear" probably resembles the more acute processes. The principal similarity is one of fibroblastic proliferation which ultimately leads to healing by scar or to formation of a framework for growth of normal tissue. Good

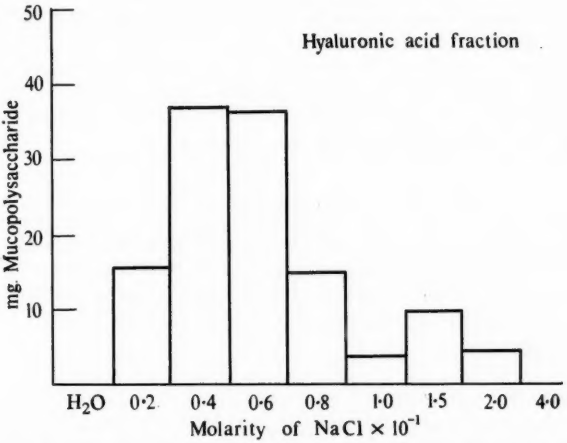

FIG. 3.—Chromatographic separation of the hyaluronic acid fraction obtained by zone electrophoresis. Two compounds were obtained, hyaluronic acid and heparitin sulphate. Refer to text for further details.

reviews (Howes, 1954; Dunphy and Udupa, 1955; and Jackson, 1958) exist for consideration of the role of connective tissue in wound healing.

It has been suggested that granuloma tissue has rather active and diversified metabolism (Gedigk and Bontke, 1957) and, as might be expected, metabolic variations seem to occur with the age of tissue (Kellgren, 1952). Observations of healing wounds and granulation tissue by histochemical means indicate an early increase in MPS content at the site of newly formed tissue (Taylor and Saunders, 1957). Thus, fibroblastic growth has a capacity for rapid formation of connective tissue components (Jackson, 1958). Further evidence of dynamic metabolism of the MPS has been shown by radioactive studies and determinations of turnover rates of the compounds in normal and disease tissue (Boström and Åqvist, 1952; Schiller, Mathews, Goldfaber, Ludoweig and Dorfman, 1955). Rapid incorporation of precursors into the MPS has been observed. One material, hyaluronic acid, even under normal conditions has been determined to have a half-life of approximately 2 days, although a somewhat slower one is found for chondroitin sulphate. Growth of fibroblasts in tissue culture further indicates the ability

of these cells to form MPS rapidly (Berenson, Lumpkin and Shipp, 1958). The capacity for rapid synthesis and formation of complex substances serves as a necessary function in growth of new and reparative tissue.

The fibroblastic proliferation, furthermore, has an ability to form a wide spectrum of chemical compounds. The isolation thus far of four different MPS from this inflammatory tissue, a mucoprotein (observations in this laboratory with Dr. A. Fishkin indicate formation of a homogenous mucoprotein which has approximately 55 per cent polypeptide and 12 per cent sialic acid and 5 per cent hexosamine), and collagen points out the heterogeneity of the composition of granulation tissue and the complexity of study of tissues involved in inflammation and repair. Identification of a group of compounds with similar but distinct chemical compositions should make one cautious in interpretation of data derived from studies of a unit common to more than one substance, for example, hexosamine or sulphate, as referred to earlier. It is apparent that although the MPS have similarities, there are pronounced individual chemical, physico-chemical and, most likely, physiological dissimilarities. Further studies are indicated to learn how and why these compounds are put together.

SUMMARY

The acid mucopolysaccharides were isolated from inflammatory tissue produced by injecting air and turpentine into subcutaneous tissue of rats. Four mucopolysaccharides were identified: hyaluronic acid, heparitin sulphate, chondroitin sulphate A and chondroitin sulphate B. Inflammatory tissue is a metabolically active and complex tissue.

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THE DISTRIBUTION OF THE BLOOD GROUP A ANTIGEN IN HUMAN TISSUES

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IT has been known for many years that blood group antigens are present in many human tissues and body fluids other than red cells and saliva. Their presence has usually been demonstrated by agglutination inhibition tests, using extracts of organs or tissues to inhibit specific agglutinating systems—for example, group A red cells and anti-A serum.

This approach has contributed little to the precise localization of these antigens within organs and tissues. More recently, however, several different methods have been used to study the distribution of blood group antigens at a cellular level.

Thus Coombs, Bedford and Rouillard (1956), have developed a specific mixed agglutination test in which tissue cell suspensions, sensitized with specific anti-A or anti-B agglutinin are mixed with human group A, B or O red cells respectively. Where the A or B antigen is present on both tissue cell and red cell, the presence of the specific agglutinin causes adherence of red cells to the tissue cell surface—the " mixed agglutination " effect. This method, therefore, allows observation of single cells, and has been used to reveal blood group antigens on epidermal cells, and on platelets (Coombs *et al.*, 1956; Coombs and Bedford, 1955).

Another recently introduced method is to absorb agglutinating antisera with a tissue cell suspension, and subsequently to elute specific anti-A or anti-B activity from the cells (Nelken, Nelken, Michaelson and Gurevitch, 1956; Berroche, Maupin, Hervier and Dausset, 1955).

A method which is particularly apt for locating tissue antigens *in situ* is that of Coons, in which a high-titre specific antiserum, conjugated with fluorescent dye, is used to " stain " fresh tissue sections. The sites of antigen-antibody reaction in the section then appear as fluorescent areas on ultra-violet microscopy. The use of this method to study blood group antigens in the epithelium of gastric mucosa and in some other situations had been described (Glynn, Holborow and Johnson, 1957; Glynn and Holborow, 1959).

The present work is an attempt to describe the distribution of the human blood group A antigen in human tissues, in terms of two of the methods outlined above—the mixed agglutination test and the fluorescent antibody technique. These two techniques, independently carried out, have revealed a remarkably close correspondence of results.

MATERIALS AND METHODS

Fluorescent Antibody Technique

Tissues.—At first, most of the tissues examined were obtained post-mortem, up to 24 hr. after death. Later, tissues were taken at autopsies on new-born infants, performed within a few hours of death, and these specimens usually yielded more satisfactory results. Tissue from 2 foetuses (about 16 and 20 weeks respectively) freshly obtained from abortions, also yielded very good results. Small blocks of unfixed tissue were placed in closed polythene containers and quickly frozen in dry-ice/alcohol mixture, and thereafter stored at −20° until examined.

Sections.—Blocks of frozen, unfixed tissue were transferred to a cryostat at −20°, in which sections were cut with a microtome, according to the technique described by Coons and Kaplan (1950). Such frozen sections, placed on slides, and thawed, were subsequently treated with anti-A globulin conjugated with fluorescein isocyanate.

Conjugate.—Rabbit antisera were prepared against human blood-Group-A substance (batch 203/112/1, prepared from ovarian cyst material and kindly given by Professor W. T. J. Morgan, Lister Institute, London). The method of immunization, using coated streptococcal vaccine, has been previously described (Glynn, Holborow and Johnson, 1957). These antisera specifically precipitated human A-substance, and gave no cross-reaction with B, H or Le^a substances.

The preparation from these antisera of fluorescent conjugate (using florescein isocyanate), the method of absorbing the conjugates to increase specificity, and the method of staining sections have been previously described (Glynn, Holborow and Johnson, 1957).

Mixed Agglutination Technique

Glassware.—Siliconed glassware was used throughout.

Saline.—Normal NaCl solution buffered at pH 7·2 by adding 10 ml. Sørensen's phosphate buffer to each litre salt solution.

Special diluent for cell suspensions.—To prevent red cells and tissue cells sticking to the glass, the cells were suspended in 1 : 200 dilution of normal rabbit serum in saline. The normal rabbit serum had previously been heated at 56° for 30min. and absorbed free of antibodies to human A, B and O red cells.

Anti-A sera.—Human anti-A serum of the so-called " immune " type (from a Group O person) was used. This serum had an agglutinin titre of 2000 for human A red cells. The rabbit anti-human-A-substance serum used in the fluorescent antibody study (absorbed with human O red cells) also gave satisfactory results. Ordinary human anti-A grouping sera was used, but gave generally weaker results. The sera were heat inactivated before use.

Preparation of tissue cell suspensions

Human tissues were obtained from surgical and post-mortem material. The post-mortem samples were generally procured within 24–36 hr. of death.

Cell suspensions were prepared either by gently scraping the tissue surface with a cataract knife or a brush, or by mincing the tissue with scissors in a small volume of the normal rabbit serum diluent. If cellular degeneration was evident, the suspension was discarded. A fine suspension could be obtained by selective use of the pipette. The cells were deposited by gentle centrifugation (1000–1500 r.p.m.) and washed ×3 with diluent. The cells could be used at this stage or after storage in diluent containing 20 per cent glycerol at −30° or −70°.

Mixed agglutination reaction

The method of testing for mixed agglutinatiou has been described previously (Coombs, Bedford and Rouillard, 1956). Suspensions of cells were obtained from various human tissues and treated as described above. The blood group of the patients was established by standard methods of red cell typing. Tissues from 9 group A patients and 5 group O patients were examined.

The mixed agglutination reaction was performed using the human " immune " anti-A serum at a dilution of 1 in 5.

RESULTS

Mixed Agglutination Technique

Cells from most of the tissues from Group A patients showed strong mixed agglutination with human A red cells—there being no mixed clumping with human O red cells. No mixed agglutination with human A red cells was obtained with cell suspensions from Group O tissues. The result of testing cell suspensions from specific tissues are given in the Table, and the typical appearances of positive and negative tests shown in Fig. 1 and 2.

TABLE.—*Distribution of A Antigen in Human Tissues*

Tissue	Mixed-agglutination method	Fluorescent anti-A antibody
Mouth (epithelium)	+	+
Tongue (epithelium)	+	+
Pharynx (epithelium)	+	+
Oesophagus (epithelium)	+	+
Stomach (epithelium)	n.t.	+*
Duodenum (epithelium)	n.t.	+
Small intestine (epithelium)	n.t.	+ (jejunum, ileum).
Large intestine (epithelium)	+ (anal canal and rectum)	+ (appendix, colon).
Salivary glands	n.t.	+ (mucous cells).
Pancreas	n.t.	+ (foetal ? islets).
Lachrymal gland	n.t.	0
Thyroid epithelium	+	0
Pituitary (pars intermedia)	n.t.	+
Adrenal (parenchyma)	n.t.	0
Kidney (collecting tubules)	+	+
(glomeruli)	n.t.	+
Ureter (epithelium)	+	+
Bladder (epithelium)	+	+
Urethra (epithelium)	+	n.t.
Trachea (epithelium)	+	n.t.
Bronchus (epithelium)	n.t.	+
Fallopian tubes	+	+
Uterus (endometrium)	+	+
Vas deferens	+	n.t.
Testis	n.t.	0
Spermatozoa	n.t.	0
Skin (epidermis)	+†	+
Liver (parenchyma)	0	0
(Kupffer cells)	+	0
Skeletal muscle	0	0
Cardiac muscle	+ (weak)	0
Parietal pleura	+ (weak)	n.t.
Parietal pericardium	+	n.t.
Vascular endothelium	+	+

n.t. = not tested.
* Glynn, Holborow and Johnson (1957).
† Coombs, Bedford and Rouillard (1956).

Fluorescent Antibody Technique

Results with this method are also given in the Table, for comparison with those obtained by the mixed agglutination technique. A brief descriptive account of the findings in the various body tissues follows.

The distribution of the A antigen in tissues may be described under 2 headings (i) its occurrence in epithelial cells, or tissues arising wholly or partly from epithelial origins, and (ii) its distribution in non-epithelial tissues.

Epithelial Tissues

Buccal epithelium, tongue, pharynx and oesophagus (Fig. 3).—These tissues have been examined only in a 20-week foetus. The stratified squamous epithelium in all these sites was positive for the A antigen in all except the deepest epithelial layer. The mucous glands in the region of the pharynx were also strongly positive.

Salivary and lachrymal glands.—The A antigen is present in considerable quantities in the mucus-secreting cells of the mixed salivary glands but absent from the serous cells (7 specimens examined). Thus, the submaxillary gland gives positive staining in regions corresponding to the mucous cells, but parotid gland is uniformly negative. Some submaxillary tissue examined has been negative, but this may be associated with the non-secretor trait. In general, the secretor status has not so far been taken into account in these studies (with the exception of stomach, see below). One specimen of lachrymal gland was negative.

Thyroid gland.—The epithelial cells of the thyroid acini have been uniformly negative in 7 specimens examined.

Pituitary gland.—Positive staining in the pituitary (2 adult specimens) is confined to the colloid and lining cells of the vesicles of the pars intermedia. Other parts of the pituitary have shown no staining.

Thymus.—In foetal tissue and in the new-born, the outer concentric cells of Hassall's corpuscles stain specifically for A substance (4 specimens). The inner degenerate cells do not appear to stain but have a pearly blue auto-fluorescence. Thymuses from older subjects have not been examined.

Adrenal.—The cells of the cortex and medulla were uniformly negative, but the lining cells of the sinusoids, and occasional isolated unidentified cells in the medulla gave specific positive staining.

Liver.—No specific staining has been found in a number of specimens of liver nor has the A antigen been detected in the epithelial lining of bile ducts or gall-bladder. The Kupffer cells appear negative.

Pancreas.—Groups of cells which appear to be developing islets of Langerhans stain specifically for A substance in tissue from foetuses and from premature or new-born infants (4 specimens). The pancreatic acinar cells themselves, and the ducts, are negative. In adult pancreatic tissue, all obtained at routine post-mortem, tissue was unsatisfactory owing to autolysis.

Stomach and first part of duodenum.—The findings here have been fully described elsewhere (Glynn, Holborow and Johnson, 1957).

Small and large intestine.—In both adult and foetal tissue strong positive staining for the A antigen is present in the goblet cells of the mucosa of the jejunum, ileum, appendix, caecum, colon and rectum.

Respiratory tract.—In the lungs the most striking positive staining occurs in the peribronchial mucous glands which lie in relation to the bronchi and bronchioles. In these glands the mucous cells (as in the salivary glands) stain brightly, while their duct linings and the lining epithelium of the bronchi themselves also stain, though rather faintly. The remainder of the lung tissue is negative, including the bronchial cartilage, except for capillaries (see below) in the vicinity

of the peribronchial glands. The alveolar capillary network appears consistently negative.

Kidney (Fig. 6).—The glomerular tufts and peritubular capillaries in infants and foetuses (4 specimens) show positive staining of the " capillary " type (see below). This has not been seen in the few adult kidneys examined, possibly due to post-mortem change. Strong positive staining is seen in the cells lining the collecting tubules throughout the kidney, and in the cells lining the calyces and pelvis. The convoluted tubules are negative.

Ureter and bladder (Fig. 4).—The transitional epithelium of ureter and bladder is positive throughout its thickness, except in the deepest cell layer.

Uterus, Fallopian tubes and ovary.—These have been examined only in foetal material (1 specimen). The endometrium and the lining epithelium of the Fallopian tubes were both positive throughout their thickness. A line of stronger staining was noted at the endometrial surface. In the ovary no definite staining was seen except of the vascular tissue.

Spermatozoa and testes.—No specific staining of sperms from 4 group A adults was found, nor in sections of 2 infant testes.

Skin and its appendages (Fig. 5).—Sections of skin (20-week foetus, 3 infants and 1 child, aged 8 yr.) show specific staining at well-defined sites. These are the ducts of the sweat glands, but not the sweat glands themselves, the ducts of the sebaceous glands, but not the sebaceous glands themselves and the stratum granulosum of the epidermis. The other layers of the epidermis (stratum Malpighii and stratum corneum) and the hairs are negative. In the hair follicles a layer of cells continuous with the stratum granulosum is positive, and in one instance positive granules were seen in the external root sheath.

Non-epithelial Tissues

Vascular endothelium.—Specific staining of capillaries has frequently been noted in a variety of organs obtained post-mortem—namely thyroid, thymus, heart muscle, pancreas, salivary and lachrymal glands, skin and subcutaneous

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EXPLANATION OF PLATE

FIG. 1*a*.—Mixed agglutination reaction between human Group O oesophageal epithelial cells and human group A red cells. Negative result—red cells have no affinity for serum-treated epithelial cells. (Phase contrast microscopy : ×407.)

FIG. 1*b*.—Mixed agglutination reaction between human Group A oesophageal epithelial cells and human group A red cells. Positive result—red cells adhering specifically to serum-treated epithelial cells. (Phase contrast microscopy : ×407.)

FIG. 2*a*.—Mixed agglutination reaction between Group O endothelial cells from vena cava and human group A red cells. Negative result. (Phase contrast microscopy : ×407.)

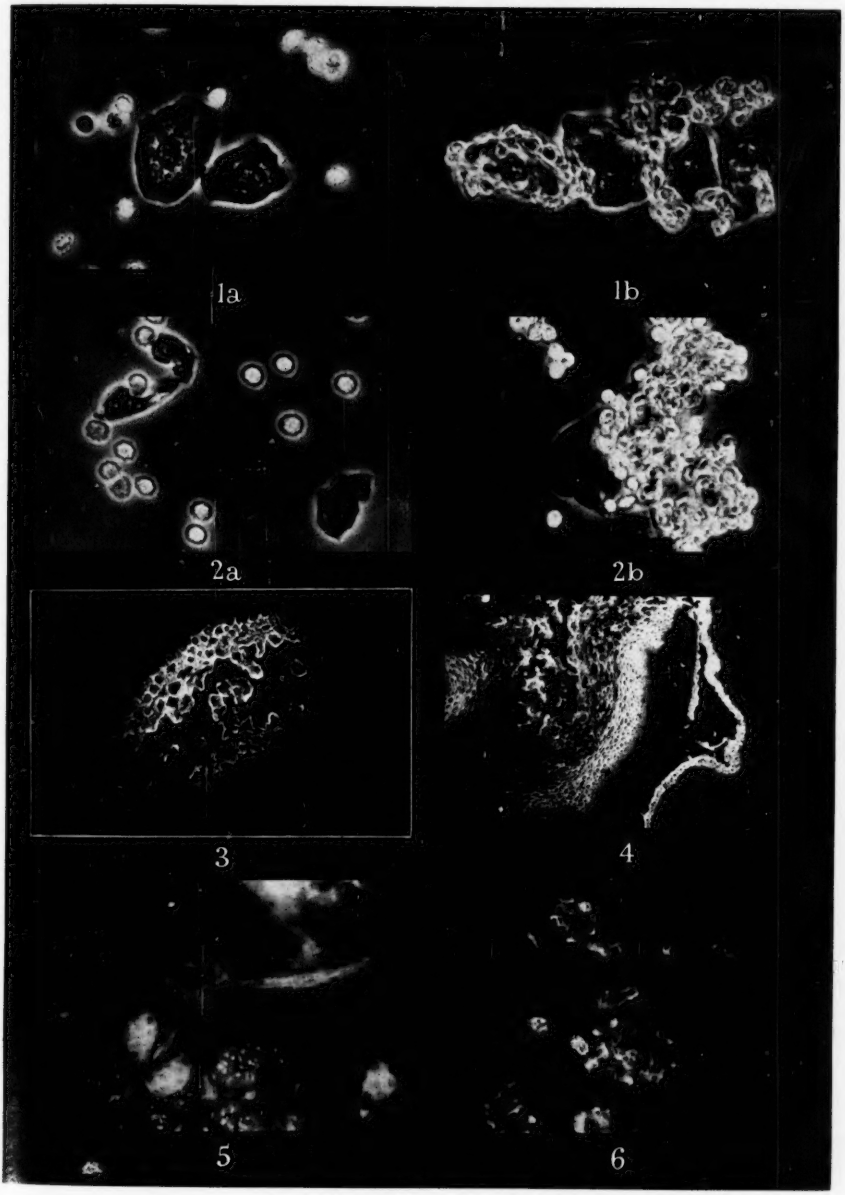
FIG. 2*b*.—Mixed agglutination reaction between group A endothelial cells from vena cava and human group A red cells. Positive result. (Phase contrast microscopy : ×407.)

FIG. 3.—Unfixed frozen section of foetal human group A oesophagus stained with anti-A fluorescent conjugate. Stratified squamous epithelium positive for A antigen. ×130.)

FIG. 4.—Unfixed frozen section of foetal bladder and ureter stained with anti-A conjugate. Transitional epithelium positive for A antigen. (×32.)

FIG. 5.—Unfixed frozen section of child's skin (aged 12) stained with anti-A conjugate. Positive staining of A antigen in capillary endothelium, and in sweat duct epithelium. Sweat gland epithelium negative. (×130.)

FIG. 6.—Unfixed frozen section of foetal group A kidney stained with anti-A conjugate. Glomeruli and collecting tubules positive for A antigen. (×130.)



Holborow, Brown, Glynn, Hawes, Gresham, O'Brien and Coombs.

tissue, stomach mucosa and kidney (both in glomeruli and in peritubular capillaries). In the foetal tissues examined the vascular endothelium of the larger vessels (including those in the umbilical cord), as well as that of the capillaries gave positive staining. In the 20-week foetus positive staining of the sinusoidal epithelium of the spleen and of the adrenal was clearly seen and also of the meningeal capillaries, the lining of the choroid plexus and of occasional blood vessels within the brain substance.

Effect of ethanol on sections

Pretreatment of sections with 90 per cent ethanol for 30 min. consistently prevented subsequent staining of vascular endothelium. Lower concentrations of alcohol (down to 45 per cent, 90 min. treatment) were also effective in abolishing staining of this type. Ethanol treatment removed all staining from spleen, adrenal, brain and thyroid, where all detectable A antigen is located in the endothelial cells lining capillaries or sinusoids. In contrast, epithelial staining is usually unaffected by ethanol, so that in the epithelium of the bladder, uterus and practically the whole lining of the alimentary tract, for example, the A antigen survives this treatment. In kidney, ethanol abolishes the glomerular staining, but leaves the collecting tubules positive, and in the thymus abolishes vascular staining, leaving the Hassall's corpuscles positive. The only exceptional epithelial tissue so far noted is the skin, where pretreatment with ethanol prevents positive staining in all the sites described.

Effect of other solvents on vascular endothelium

Chloroform treatment of sections completely prevented subsequent staining, and chlorobenzene almost completely prevented it. Acetone and ether were without effect.

Effect of ethanol on cells tested by the mixed agglutination method

Cells from the kidney pelvis, oral cavity, bladder, oesophagus, trachea and inferior vena cava endothelium were treated with 70 per cent alcohol for 40 min., and washed. All gave positive mixed agglutination tests with anti-A serum after this treatment.

DISCUSSION

Although both methods of detecting the A antigen have not been applied to all the tissues examined, both tests have been done on enough tissues to indicate close agreement between them. The results show that in group A individuals the A antigen is widely distributed in 2 principal sites—epithelial tissue and vascular endothelium.

The lining epithelium of the alimentary tract is strongly positive for the A antigen in all sites that have been examined (mouth, pharynx, oesophagus, stomach, duodenum, jejunum, ileum, appendix, colon, rectum and anal canal). The precise localization of the antigen is well shown by the fluorescent antibody method. In the columnar epithelium of the small and large intestine A substance is most conspicuous in the mucous droplets of goblet cells, although fainter

staining of indifferent epithelial cells may be seen, particularly at their luminal ends. The stomach and duodenum show special features in their distribution of blood group antigen (Glynn, Holborow and Johnson, 1957).

The stratified squamous epithelium of mouth, pharynx and oesophagus differs in that the positive staining for the A antigen is not cytoplasmic, but appears to outline the cells, either in the cell membrane or in the intercellular substance. In contrast, in the mucus-secreting cells of the mucous glands of the mouth and pharynx, and of the sublingual and submaxillary salivary glands, the cytoplasm is strongly positive. Both methods show that the tracheal and bronchial epithelium possess the A antigen, but again the cells of the peritracheal and peribronchial mucus-secretory glands show the strongest cytoplasmic fluorescent staining.

The stratified squamous epithelium of the skin gives a somewhat different picture. Although the epidermal cells from the surface give a positive mixed agglutination test (Coombs, Bedford and Rouillard, 1956) the fluorescent method shows detectable antigen only in the granular-cell layer, in the sweat and sebaceous ducts, and in one cell layer of the hair follicles. This partial discrepancy between results by the two methods may be due to quantitative considerations discussed below.

In the urinary tract, A substance is detectable throughout the lining epithelium, extending from and including the collecting tubules in the kidney to the urethra. The transitional epithelium shows the non-cytoplasmic, intercellular type of staining seen in squamous epithelium, involving all layers except the Malpighian layer.

These results on epithelial tissue, particularly squamous and transitional epithelium, must be interpreted cautiously in view of the possibility that blood group substances may have been adsorbed by the cells from the secretions (saliva, urine, meconium) bathing them. When pharyngeal epithelial cells from a group O subject were incubated at 37° for 30 min. with boiled group A secretor saliva, and then washed twice in saline, they did not become positive in the mixed agglutination test with anti-A sera. Cells from group O bladder and ureter, however, did become positive on this treatment. Fluorescent anti-A serum stains the epithelium at these sites brightly and uniformly through several cell layers, and it is difficult to envisage how absorption of the very small amount of blood group substance in urine could give rise to such deposition of A antigen.

The presence of A antigen in vascular endothelium, which was previously reported (Glynn and Holborow, 1959) has been confirmed in a wide variety of tissues. The mixed agglutination method has been applied chiefly to endothelium scraped from the inferior vena cava, which has proved consistently positive in group A subjects. By the fluorescence method specific positive staining is seen chiefly in capillary endothelium, but also in the intima of the blood vessels of many tissues. The kidney glomeruli in new-born infants are conspicuously positive in this respect. The lung capillaries, however, have been negative in material strongly positive elsewhere. The ethanol solubility of this vascular antigen has been very strikingly consistent in the fluorescence study; that endothelium of the inferior vena cava remains positive in the mixed agglutination method after ethanol treatment suggests that the amount of ethanol-insoluble antigen present in endothelium may be too small for detection by fluorescent antibody. The same quantitative considerations may apply also to the A antigen in the cells of the epithelium.

Two discrepancies between the methods were found. Thyroid epithelium and Kupffer cells, both positive by the mixed agglutination method, were negative by the fluorescence method. It is possible that contamination of the cell suspensions with capillary endothelium contributed to this finding.

SUMMARY

The distribution of the blood-group A antigen in a variety of human tissues has been studied by 2 methods—the mixed agglutination method, using human immune anti-A serum, and Coons' fluorescence method, using a conjugate of rabbit antiserum to human A substance. Good agreement in results was found between the methods, which show that the A antigen is widely distributed in human tissues, principally in epithelial tissues and in vascular endothelium.

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THE EFFECT OF TRITON, A SURFACE-ACTIVE POLYOXYETHYLENE ETHER, ON THE DEVELOPMENT OF EXPERIMENTAL SILICOSIS

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IN 1951 Cornforth, Hart, Rees and Stock reported on the antituberculous activity in mice of a commercial surface-active agent, non-ionic Triton WR 1339, and of other similar macromolecular compounds. Their chemical structure is shown in Fig. 1.

Because of the well-known clinical association of tuberculosis with silicosis, Rees (1955) conducted an experiment in which he studied the effect of Triton on

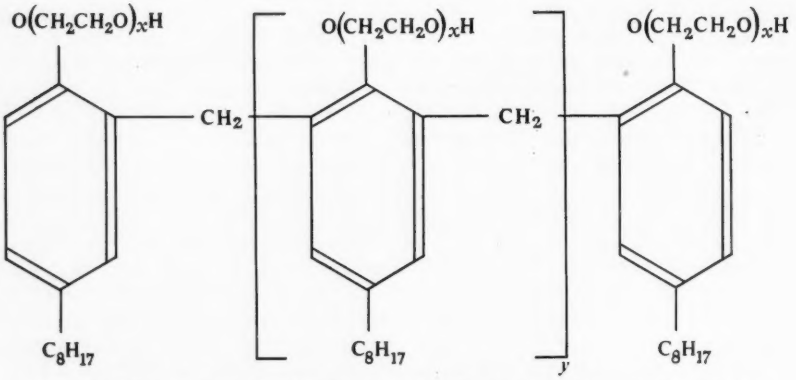

FIG. 1.—Linear macromolecular structure of surface active polyoxyethylene ethers, $y = 0$ upwards. $x = 10$–90.

the development of silicosis in mice which had been injected intravenously with finely particulate quartz. The rationale of the work was based on the surface active theory of silicosis, and it was anticipated that these surface active agents might in some way alter the surface properties of quartz particles, or modify the nature of adsorbed materials. Rees assessed the result by comparing histological sections of the whole liver of Triton treated and non-treated mice which had received quartz intravenously. While no obvious differences were observed in the size, morphology or maturation of the lesions within the two groups, it was noted that the liver sections of the treated animals appeared to be up to twice the size of the non-treated. This observation was confirmed by one of us (C.V.H.), and it seemed possible, therefore, that the greater bulk of liver tissue in the treated animals might contain more lesions for the given dose of quartz. This work was discussed by Panel 3 (Biological Effects of Dust) of the Medical Research Council's

Industrial Pulmonary Diseases Committee, and it was decided to repeat the experiment by intratracheal injection of powdered quartz into the lungs of rats, some of which should be treated with Triton, and to assess the results by collagen analyses as well as by histology. It was planned to start treatment immediately after the injection of the dust. Groups of animals were to be treated with quartz alone, with quartz plus Triton, and with Triton alone.

TABLE I.—*Analysis of Quartz Dust Used for Intratracheal Injection of Rats*

Size (μ)	Number (per cent)	Mass (per cent)
0·9–1·3	24·6	5·9
1·3–1·8	52·2	35·7
1·8–2·6	19·3	37·0
2·6–3·6	3·9	21·4

Mean diameter, 1·9 μ; specific surface, 1·6 m.^{2}/g.; SiO_2, 98·1 per cent; ignition loss, 0·56 per cent; residue after HF, 1·4 per cent.

MATERIALS AND METHODS

Animals.—M.R.C. black and white male hooded rats (average body weight 150 g.) were used.

Quartz.—A crystalline quartz dust with a free silica content of 98·1 per cent and a size distribution as shown in Table I was used. The dose of this dust was 50 mg. per rat. The dust was suspended in 0·9 per cent saline in a concentration of 50 mg./ml. and autoclaved.

Triton WR 1339.—The Triton was supplied through the courtesy of the Bayer Company. It was made up in aqueous solution, 12·5 per cent, and was prepared for us by Dr. Rees. The dose used was 125 mg./kg. twice weekly, injected subcutaneously into the skin of the lateral abdominal wall.

Injection technique.—The animals were lightly anaesthetized with ether, and the quartz suspension introduced into the lungs from a syringe through a 6 in. × 16 gauge blunt needle which had been inserted into the trachea under direct vision.

Histopathological technique.—The animals were killed with coal gas, and the lungs injected *in situ* with about 10 ml. of 15 per cent formol-saline. The animals were then autopsied and the unopened lungs preserved in 15 per cent formol-saline. After fixation the lungs were sectioned in the sagittal plane near the hilum, and whole lung blocks were embedded in paraffin. Five sections were cut from each block. Of these one was stained with haematoxylin and eosin, and another impregnated with silver after the method of Gordon and Sweet. All trimmings were saved, and blocks and trimmings were deparaffined and added to the remains of the lung.

Collagen analysis.—The method used was essentially that of Stacey and King (1954), which depends on hydrolysis of the proteins, followed by colorimetric determination of hydroxy proline, an amino acid of high concentration in collagen but present in very small amount in other proteins. The lungs were dried to constant weight, freed of fat by alcohol–ether extraction and reweighed. They were then ground in a steel mortar until all the powder would pass a 60 mesh sieve. Two samples of between 15 and 25 mg. from each lung were hydrolysed in sealed Pyrex tubes with 6 N–HCl. The whole hydrolysate was treated with activated charcoal to remove tyrosine which interferes with the hydroxy proline determination, and filtered into 25 ml. volumetric flasks, followed by 3 washings with 6 N–HCl. The flasks were then made to volume with more 6 N–HCl. Hydroxy proline was estimated in duplicate samples of this by the method of Stacey and King (1954).

RESULTS

All the animals but 3 survived the immediate post operative period. During the 205 days of the experiment 7 more died; the rest were killed at various intervals, as set out in Table II.

TABLE II.—*Collagen in the Lungs of Rats Treated with Triton, with Quartz, and with Quartz plus Triton.*

Days of survival after injection	Treatment: Triton — Total collagen both lungs (mg.)	Triton — Per cent collagen in dried lung	Quartz — Total collagen both lungs (mg.)	Quartz — Per cent collagen in dried lung	Quartz plus Triton — Total collagen both lungs (mg.)	Quartz plus Triton — Per cent collagen in dried lung
10	14	6·4	93	10·8	28	6·3 a
			60	15·0	38	9·6
					93	22·6
20	15	5·7 a	58	7·6 a	26	63
					49	9·3
40	16	5·4	27	4·5	47	8·4
					97	14·4 a
60	—	—	—	—	40	7·5
					285	37·1 a
70	16	4·1	54	7·3	37	5·0 a
					125	10 a
80	—	—	—	—	172	7·4 a
90	17	4·4	55	7·1	62	7·0 a
					117	9·5
					188	14·7
130	23	6·4	185	13·1	168	8·3
160	29	6·0	483	27·2 a	248	20·1
	24	5·5	356	29·3	278	24
					375	26·5
205	21	6·2	143	15·5	153	8·1
	31	7·2	367	34 a	250	13·7
	36	8·8			254	14 a
	47	7·3				

a = Abscess in lung.

In the group receiving Triton alone no animals died during the experiment, and no pathological changes were seen in the lungs. In the lungs of quartz injected rats, whether Triton treated or not, small, approximately 1 mm. grey-white flecks were visible in the pleura, and less easily in the parenchyma of the lungs at 10 days. These were formed of focal collections of macrophages in which there was a fine reticulin network. At 20 days the nodules were slightly larger, loosely arranged, but with slightly more reticulin, although still only of grade 1 fibrosis (Belt and King, 1945). From this time onwards individual lesions tended to be about 2–5 mm. in size, becoming firmer and paler, although in some animals there were areas where these lesions were confluent, more especially in the later stages of the experiment. Confluent lesions up to 1·0 cm. were seen. Microscopically, at 40 days the lesions were cellular, with a dense reticulin network of grade 1 fibrosis. At 70 and 90 days there was some diminution in cellularity with well-developed collagen fibres to the level of grade 3 fibrosis. At 130 days the lesions were almost acellular, with hyalinizing centres, and were completely collagenized—grade 4 fibrosis. Grade 5, confluent, acellular, hyalinized lesions (Fig. 2 and 3) was reached at 160 days although one animal killed at 205 days showed only grade 4 fibrosis. In 3 animals who had abscesses in their lungs there were silicotic nodules with the same degree of fibrosis seen in other animals at the same time. Abscesses appeared in occasional animals in both the quartz group and the Triton-treated quartz group: they were not appreciably different

in size or appearance. Their walls were composed of dense fibrous tissue, and areas of bronchiectasis were usually evident elsewhere in the lungs.

In the animals dying other than post-operatively, or with abscesses, no pathology was found at autopsy except the silicotic changes in the lung. Frequent cultures were made for pathogens, *i.e. Salmonella*, but none were found. Treatment with Triton had no observable effect on the development and progress of the lesions as determined by gross and microscopic examination; while variations could be seen between animals in the 2 groups killed at the same time, these sometimes favoured one group and sometimes the other. On the basis of the grading of fibrosis the Triton-treated quartz animals generally attained a particular grade slightly sooner than the quartz-alone-animals. But there was an equally steady increase in the maturity of the collagen in both groups.

Collagen analysis.—The collagen content of the Triton control animals was within the expected normal range (5–8 per cent) and showed the usual overall small increase with time. The collagen content of the animals receiving quartz showed large individual variations, irrespective of treatment with Triton. Some of these variations can be attributed to the presence of abscesses in the lungs. On theoretical grounds one would expect the abscesses to give increased collagen contents due to their fibrous capsules. But if, on the other hand, the process of abscess formation results in the necrosis of already formed silicotic nodules and escape of the silica and pus from the lung, a lower result would be anticipated. In either instance one does not know what the effect of the inflammatory process responsible for the abscesses is upon the development of silicotic nodules. An overall inspection of the figures for the collagen content of the quartz and quartz plus Triton groups, expressed as total collagen and as a percentage of the dry fat-free lung weight, suggests that there is no significant difference between them (Table II). There is no apparent reason for the unusually high value of 93 mg. (22·6 per cent) for one of the quartz Triton animals killed at 10 days, nor can any explanation be provided for drops in collagen content at 205 days seen in both groups.

In order to determine whether the differences between the collagen analysis of the quartz group and of the quartz plus Triton group were significant we made the statistical comparison shown in Table III.

In view of the possible effects of abscesses on the determined collagen content of lungs, it seemed best to exclude results on these lungs from the calculations.

TABLE III.—*Table of Means and Differences between Means for Collagen in Rats Treated with Quartz and with Quartz plus Triton, with the Estimated Standard Error of Each Difference, and t.*

Days	Quartz	Quartz and Triton	Difference	S.E. of Difference	t	p
10	76	72	−4	36·2	0·11	>0·9
20	..	38	..	..	..	..
40	27	47	+20	44·4	0·45	>0·6
60	..	40	..	..	..	..
70	54	..	..	..	..	..
80	..	..	..	..	..	..
90	55	153	+98	44·4	2·2	0·05
130	185	168	−17	51·2	0·33	>0·7
160	356	280	−76	33·1	2·2	0·05
205	143	202	+59	44·4	1·3	0·2

Differences between mean collagen content are shown for quartz treated animals compared with those treated with quartz plus Triton at different killing periods. To test the significance of the difference between means the ratio of the difference to its estimated standard error was determined (t in Table III). The probability that this ratio was not due to chance was read from a t distribution table (Fisher and Yates, 1948). At two killing periods t was 2·2 ($p = 0{\cdot}05$), namely at 90 and 160 days. However, since one of these is positive and the other negative, it is unlikely that the differences could have been due to Triton. The statistical analysis is fully set out by one of us (Byers, 1959).

SUMMARY

On the basis of gross and microscopic pathology, and of collagen analyses, it is concluded that Triton does not have any effect on the course of experimental silicosis produced in rats by intratracheally injected powdered quartz dust.

We should like to thank the Medical Research Council and the National Coal Board for financial assistance. One of us (P. D. B.) held the Allan Blair Memorial Fellowship of the Canadian Cancer Society.

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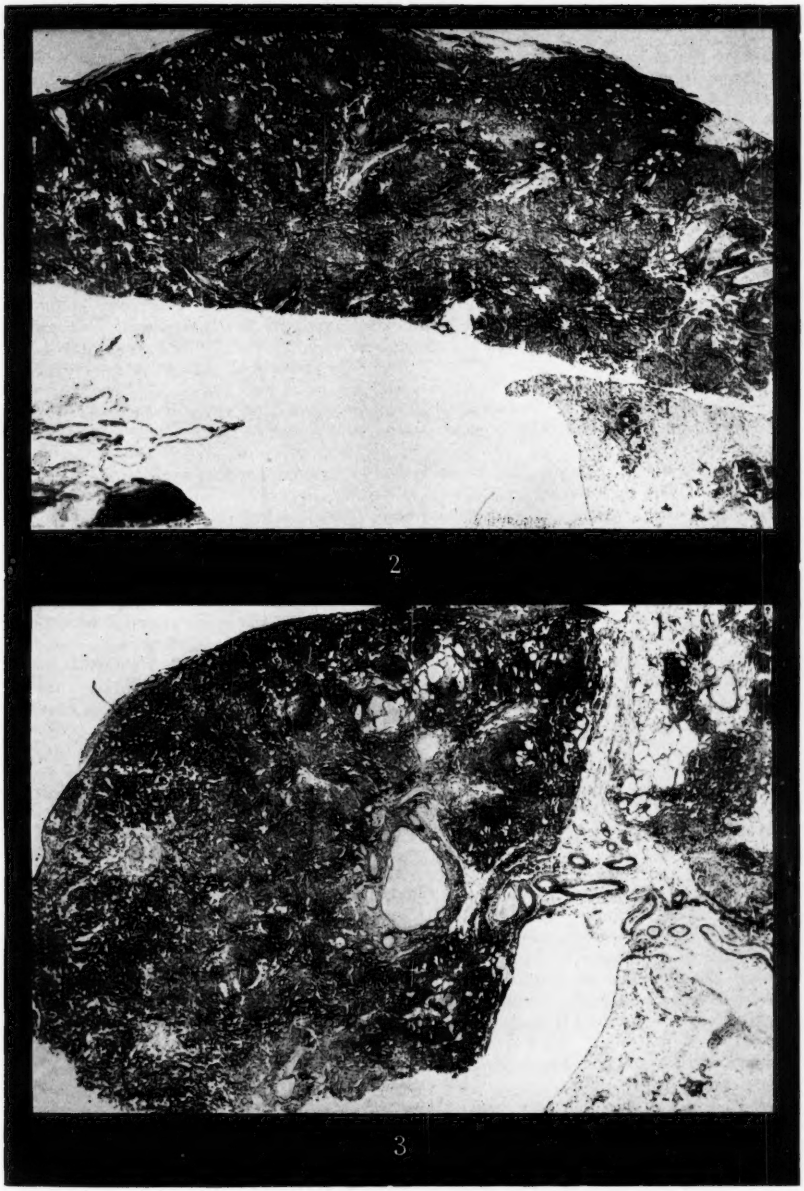
EXPLATION OF PLATE

Fig. 2 and 3.—Grade 5 fibrosis in the lungs of rats, 205 days after the intratracheal injection of 50 mg. of quartz. Silver impregnation × 16. (2) Upper, quartz alone ; (3) lower, quartz followed by twice weekly subcutaneous injections of Triton.



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3

Byers, King and Harrison.

SURVEY OF PAPERS

DAVIES and LOWE precipitated egg-albumin with anti-egg-albumin-rabbit-serum. The washed precipitates were suspended in guinea-pig serum and these suspensions were injected intradermally into guinea-pigs in which pontamine sky blue was circulating. Markedly increased capillary permeability resulted and the responsible factor was not inactivated by Mepyramine, nor did it have the biological activities of a kinin. When the antigen-antibody precipitate was deposited from the guinea-pig serum after interaction, the permeability factor remained in the supernatant. A similar factor was produced simply by acidifying guinea-pig serum at pH 5·5, and also by fractionating it in a column of diethylaminoethyl cellulose. The characters of the factors are compared with those of others described in the literature (p. 335).

IRMGARD ZEISS, NISBET and BARBARA HESLOP studied the vascularization of autologous and homologous bone grafts in rats. Cortical bone grafts from the rats' tibiae were implanted and examined subsequently at frequent intervals. The rats received an intra-arterial injection of Berlin blue and thick sections were prepared. Blood vessel counts were performed and the results statistically analysed.

The authors found that the bone implants were vascularized mainly by ingrowths of host blood vessels but there was some anastomosis between the host and graft vessels. In homografts, however, many of the blood vessels were destroyed by a homograft reaction between the 7–12th day.

The most important factor in the vascularization of both types of graft was the presence of granulation tissue which occurred maximally in the concavities and hollows of the bone. It was found that the autografts became vascularized more rapidly and more effectively than the homografts (p. 345).

ANDERSON, GOUDIE and KATHLEEN GRAY have demonstrated that the precipitating antibodies to thyroglobulin demonstrable in many cases of Hashimoto's disease are true auto-antibodies, but that the amount of thyroglobulin extractable from the patients thyroid gland may be so diminished that, in some cases, it fails to act with any Hashimoto serum.

Extracts were prepared from 12 surgically excised thyroid glands and the autologous precipitin reaction was demonstrated directly in 6 cases. No reaction could be obtained in 5 cases and it is suggested that the failure of these Hashimoto thyroid extracts to behave as antigen was due to diminution of the thyroglobulin content with, in addition, blocking of the remaining antigen by auto-antibody (p. 364).

MARGIT HAMOSH and SHAPIRO have studied the mechanism of glycogenolysis which followed endotoxin poisoning of rats. In liver tissue the breakdown of glycogen has been attributed to the increased liver phosphorylase activity resulting from an increase in phosphorylase-kinase (p. 372).

HEPTINSTALL and BRUMFITT produced pyelonephritis in rats by occluding the ureters for 24 hr. and injecting *Bact. coli* intravenously. After 6 weeks or 3 months had been allowed for healing, one ureter was again occluded for 48 hr. in some of the rats before the animals were killed and counts made of the organisms in the kidneys. The second ureteric occlusion reactivated infection, as judged by increased bacterial counts compared with controls, in most cases at 6 weeks and in some at 3 months. Increased counts were associated with abscesses near scars of the initial infection, and the histological features are described (p. 381).

WATSON and KENNEDY investigated the regeneration of tissue mast cells following their disruption by a histamine liberator, compound 48/80.

Compound 48/80 was injected intraperitoneally into rats and the animals showed the manifestations of histamine release. This was followed by i.p. injections of ^{35}S-labelled sodium sulphate. Histological preparations stained by toluidine blue and autoradiographs were prepared. The mast cell regeneration started 24 hr. after the cessation of the injections and was complete by about the 14th day. It was found that this regeneration occurred diffusely throughout the tissues and was not restricted to the perivascular areas.

The sulphur uptake gave less reliable results and this may be due to the utilization of sulphur for heparin formation and not for histamine production (p. 385).

STEWART investigated the production of intimal deposits of lipoid in the isolated aorta of rabbits. The aorta was dissected free and connected by a cannula to a pump whereby a number of differing fluids could be pulsated against its intimal surface.

It was found that cell free plasma and serum of normal rabbits did not produce deleterious effects, but the pulsation of low density lipoprotein from the serum of cholesterol fed rabbits produced lipoid deposits in the intima. Similar deposition occurred following the pulsation of fluid extracts of liver and adrenal cortex from cholesterol fed rabbits.

These intimal deposits had features in common with the early stages of atheroma and the author concludes that this atheromatous deposition can be caused primarily by abnormal low density lipoprotein in the serum (p. 389).

GRESHAM and HOWARD investigated the production of atherosclerosis, thrombosis and obesity in rats fed on a diet of 5 per cent cholesterol, 2 per cent cholic acid, 0·3 per cent thiouracil and variable proportions of butter and arachis oil.

Rats fed on a diet containing 40 per cent butter had a high incidence of myocardial and renal infarction with thrombosis and abnormal electrocardiograms.

Rats fed on the same basic diet with 40 per cent arachis oil showed extensive atherosclerosis but no thrombosis.

Rats fed on 40 per cent butter and 40 per cent arachis oil alone became obese but showed no thrombotic or atherosclerotic manifestations. They concluded that, in rats, thrombosis, atherosclerosis and obesity are all independent phenomena (p. 395).

NIFFENEGGER and YOUMANS injected tubercle bacilli of strain H37Rv intravenously into mice (Strong A), 1·0 mg. /mouse. Some of the infected mice had received, 1 or 28 days previously, 25 mg. Macrocyclon intraperitoneally ; some had received 1·0 mg. of freeze-dried BCG 28 days previously, and others were untreated. Counts of viable tubercle bacilli were made on the homogenized lungs and spleens of individual mice killed in groups at successive intervals after infection. They found that prophylactic Macrocyclon checked the multiplication of tubercle bacilli in the organs of mice as did BCG (p. 403).

HART and REES injected mice intravenously with tubercle bacilli (strain H37Rv). A dose of 0·1 mg. of bacilli caused acute infection ; 0·02 mg. caused chronic infection, predominantly of the lungs. Macrocyclon (25 mg. twice weekly) was given subcutaneously to about half the mice ; the first dose was given 2 days after the bacilli in acute infection, 60 days in chronic. Counts of stainable and of viable bacilli were made on the homogenized lungs of each of the mice, which were killed in groups at various time intervals. In any given mouse, treated or untreated, stainable and viable counts were approximately the same. Macrocyclon prevented the increased count found in the untreated acute disease. In chronic disease the count remained stable without treatment and Macrocyclon was without effect (p. 414).

BERENSON and DALFERES have characterized 4 mucopolysaccharides which have been isolated from granulation tissue experimentally induced in rats. The significance of cognate biochemical intermediates in relation to repair and defence mechanisms has been discussed (p. 422).

HOLBOROW, PATRICIA BROWN, GLYNN, MARY HAWES, GRESHAM, O'BRIEN and COOMBS tested human tissues for the presence of blood group A antigen. They used 2 methods—mixed agglutination and staining with fluorescent antiserum. The results of the 2 methods agreed well and showed a wide distribution of the antigen especially in epithelial tissues and vascular endothelium (p. 430).

BYERS, KING and HARRISON produced silicosis in rats by introducing a suspension of quartz dust into the lungs by intratracheal injection. The animals were treated with the surface active agent Triton WR 1339 by twice weekly subcutaneous injection. The work was designed to show the effects of Triton on the development of experimental silicosis. The conclusion reached was that Triton had no effect upon the natural course of this condition (p. 438).

ERRATUM

Vol. XLI, No. 3, June 1960.
(J. F. Boyd and A. N. Smith).
P. 267. Summary. Para 4 should read:

"The homograft survival time can be somewhat extended by depleting the donor rat as equally as by depleting the recipient rat."